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14. ABSTRACT
Over the first 12 months of this award, we have focused on developing an in-depth understanding of the immune system in the setting of the tumor microenvironment. We have made progress in developing methods to better analyze the relationships between primary tumor and metastatic growths with their surrounding microenvironments, and implications for immune response and dendritic cell function *in vitro* using 3D microculture techniques. We have further investigated the roles of specific cytokines on immune signaling and tested alternative methods for tumor eradication using combinatorial drugs in attempts to restore/enhance the immune response. Additionally, we have begun preparing breast cancer cell lines to aid quantifying progress in upcoming *in vivo* studies using mouse models. We anticipate that the progress made in the first 12 months will lead to combining observations into *in vitro* and *in vivo* models to better test our combinatorial immunotherapeutic strategies, restore dendritic cell function in cancer, and identifying novel tumor-associated stroma targets.

15. SUBJECT TERMS
Breast Cancer, immunotherapy, tumor microenvironment, dendritic cells, metastasis, cancer stroma.

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INTRODUCTION:

Both the immune system and cancer are complex biological systems, so restoring immune dysfunction that accompanies cancer is no simple task. While there have been recent successes in cancer immunotherapy such as PROVENGE, a dendritic cell based vaccine for prostate cancer, and ipilimumab, an anti-CTLA-4 antibody for melanoma, these have produced modest (median 4 months) survival benefits. Thus, the intimate relationships between cancer cells, immune cells, and tumor associated stromal cells must be explored and investigated in order to truly have an effective immunotherapy for breast cancer. It has more recently become clear that not only does the immune system respond to tumor cells, but the process goes both ways, with cancer also able to suppress the host immune system. Tumor-infiltrating lymphocytes (TILs) have been shown to be functionally impaired in many cancers (*1*). In tumors where TILs were found to be functional, the prognosis was consistently favorable (*2, 3*). The collective data suggest that T cell infiltration—when functionally active—leads to a favorable outcome in breast cancer. These data concerning the tumor environment complement our own findings that changes in immune cells in tumor-draining lymph nodes (TDLNs) strongly correlate with clinical outcome in breast cancer (*4*). Despite the complexity, certain elements can be teased out and focused upon for maximal impact. Our previous studies have led to key insights into the mechanisms behind the immune dysfunction that breast cancer causes. Comprehending how the different phases—activation, expansion and effector functions—of a normally functioning immune system are disrupted in the presence of breast cancer will allow us to develop strategies to counteract the problems and restore immune function to optimal levels in patients. This focus on unraveling the dynamics between breast cancer and host immune system in a comprehensive and systematic manner is the underlying principle of my goal to develop 'rational combination immunotherapy' for breast cancer, one that is truly effective long term at eliminating metastases and thereby preventing relapse in breast cancer patients. In order to build on the observations my lab has made with regards to cancer-dendritic cell function and immune cell-cancer relationships, immune responses within the tumor microenvironment must be analyzed in depth to identify unique markers, molecular and cytokine signals, and associated cell populations which may be aiding in immunosuppression. To this end, over the last 12 months I have established a strong research team. This includes staff scientist Dr. Brile Chung, PhD postdoctoral fellows Dr. Dobrin Draganov and Dr. Neta Zuckerman, research associates John Murad and Grace Jimenez. We worked closely with the CoH IRB office on a human subject's protocol which has been approved. In this first annual report, I will discuss the foundation being laid toward the goals outlined in our statement of work, and the approaches that we will test to remedy the global immune dysfunction in breast cancer.

BODY:

Over the past 12 months, I have recruited talented individuals to carry out the projects and tasks at hand. This includes staff scientist Dr. Brile Chung, PhD postdoctoral fellows Dr. Dobrin Draganov and Dr. Neta Zuckerman, research associates John Murad and Grace Jimenez. We worked closely with the CoH IRB office on a human subject's protocol which has been approved. We have built collaborations with breast cancer surgeons, pathologists, and the tissue bank at CoH in order to procure breast cancer tissue, lymph node, and blood samples for our analyses. While animal work has not yet begun, we have one approved animal protocol (IUCAC #12001) and a second protocol pending final approval (IUCAC #13042). Our team has been working hard on establishing cell lines, protocols, and preliminary experiments to build a solid foundation and direction for progressing towards the tasks in our statement of work. Listed below are the main aims which we proposed, corresponding tasks from our statement of work, and our progress to-date.

- **Aim 1. Enhance efficacy of dendritic cell (DC)-based vaccination by promoting DC maturation and clustering in vivo.**
  Task 1. Identifying mechanisms by which breast cancer disrupts DC clustering and maturation : months 1-48.
  Task 2. Testing novel strategies to enhance DC clustering *in vivo*: months 1-48.

- **Aim 2. Enhance T cell function *in vivo* by restoring immune signaling**.
  Task 3. Investigating mechanisms by which chronic IL6 affects immune function: months 1-36.
  Task 4. Testing the effectiveness of IL6-blockade plus IL27 treatment in reversing chronic IL6-induced T cell dysfunction: months 12-48.

- **Aim 3. Select optimal integrated immunotherapy combinations in animals for clinical development.**
  Task 5. Select Optimal Integrated Immunotherapy Combinations in Animal Models for Clinical Development: months 12-60.

Results:

<u>Aim 1. Enhance efficacy of dendritic cell (DC)-based vaccination by promoting DC maturation and clustering *in vivo.*</u>

- **Task 1. Identifying mechanisms by which breast cancer disrupts DC clustering and maturation: months 1-48.**
  a. Test effects of known breast cancer secreted molecules on DC clustering and maturation (month 1-36).
  b. Identify novel breast cancer secreted molecules on DC clustering and maturation using proteomics and gene expression analysis after laser capture micro-dissection (month 1-48).

In order to gain knowledge on the molecules and factors which influence DC clustering and maturation, especially in the context of cancer, we first would need to establish a better understanding of the dynamics between immune cells and the tumor microenvironment. To this end, we have worked to establish an *in vitro/ex vivo* model which helps mimic cell-cell interactions in a 3D microenvironment. It has been established in recent years that 3D cell culture environments provide a more physiological representation of cell interactions. #D cultures also produce markers and behavior that might not be seen on traditional monolayer culture experiments, and applies also specifically to the breast cancer setting (*5*). The tumor microenvironment is comprised of heterogeneous populations of cells including cancer, immune, and tumor associated stromal cells

to name a few. Clinical data and experimental models have shown that the extent and nature of immune infiltrations into tumors is an important independent prognostic factor (*6*). Recent findings suggest that tumor associated stroma is another important regulator of tumor growth and progression which may also modulate the recruitment, activation status, and retention of immune cells in the tumor microenvironment (*7*). Therefore, targeting the tumor associated stromal cells plus cancer cells are essential for the success of cancer immunotherapy.

Progression of tumor growth and initiation of metastasis is critically dependent on the reciprocal interactions between cancer cells and tumor associated stroma. Tumor associated stromal cells have been known to promote inhibitory effect on T cells by producing various factors and cytokines such as TGF beta, VEGF, HGF, IL-6, and IL-17 (*8*). These factors also may play a very important role in understanding the characteristics of DC's in the tumor microenvironment. Here, we have developed 3D cell aggregate culture system that recapitulates the human tumor microenvironment by incorporating essential populations of tumor-associated stromal cells to investigate the dynamic relationship between the tumor microenvironment and the immune system. Our novel approach focuses on separate expansion and later re-aggregation of the subpopulations of primary human breast cancer stroma and immune cells that are critical for supporting or inhibiting cancer growth. A major advantage of our tumor aggregate system is that cancer, stromal, and immune cells can be easily manipulated *in vitro* (etc. numbers, mixture of ratio, or engineered) (**Figure 1: Experimental Design**).

Generation of breast tumor-associated stroma:

To develop a system that will allow manipulation of components of the breast tumor microenvironment, specific culture conditions that support the separate ex vivo growth of breast tumor associated stroma was established. Human breast tumor tissue from patients ranging from ages 40 to 70 years was mechanically dissociated into tiny tumor fragments (approximately ≤ 1 mm diameter) and plated directly onto gelatin coated culture flasks in medium containing recombinant human EGF (100 ng/ml). Over the next 2-3 weeks, monolayers of stroma were generated and continued to expand by outgrowth from the adherent fragments (**Figure 2A**). Immuno-phenotypic characterizations of the monolayers generated in stroma cultures after 3 weeks, demonstrated the cell surface marker expression of mesenchyme/mesenchymal stem cells, GD2+, CD105+, CD44+, CD140a+ (PDGFRα+), and CD326+/- (Ep-Cam) (**Figure 2B**). Within the primary breast turmor-derived stromal cells growing in monolayer condition, we detected the basal level of epithelial marker expression (CD326). Given that 3D cell culture system provides recapitulation of both normal and pathological tissue microenvironments, we generated a novel tumor-derived 3D cell aggregate that provides spatial architecture. Shown in **Figure 2C** is a human breast stromal aggregate generated from the monolayer of cells from tissue culture. Based on previous reports, culturing both adherent and non-adherent cells in 3D allows formation of tissue structures that promotes appropriate cellular signals. For example, recent studies by Mohtashami *et al* have shown that expression of two critical Notch ligands for T cell development and differentiation, Delta-like 1 (Dll1) and 4 (Dll4), are only detected in aggregated 3D structure of stromal cells and lost upon monolayer culture (*9*).

Histological analyses of breast tumor aggregates and stroma:

Histologic analysis of human breast tumor aggregates from tissue culture at 7 day showed breast cancer cells positive for epithelial specific cytokeratin (**Figure 3A**). One of the main advantages of using our aggregate system is that each aggregate can be prepared for histological analyses. Based on comparison, immunohistochemistry revealed that a higher frequency of cytokeratin positive cells detected from the breast tumor aggregate than the aggregate comprised of human thymic stromal cells. Based on the morphologic data (**Figure 3A**), the cytokeratin expressing epithelial cells were surrounded by the cancer-associated stromal cells.

Given that the composition of the stroma in general primary tumor microenvironment can be as high as 80 percent, we further analyzed the phenotypes of cancer-associated stroma via flourescent immuno-

histochemistry. Shown in **Figure 3B** is the fluorescent immuno-histological analyses of a primary breast tumor tissue section. Approximately 60 percent of the cell population is stromal cells surrounding Her2+ cytokeratin+ breast cancer carcinoma, further demonstrating their presence in the tumor microenvironment. Next, we stained the previously generated cultured tumor stroma aggregate sample with the antibody directed against one of the mesenchyme-fibroblast markers, CD140α. As we expected, the majority of the cells surrounding the cytokeratin positive carcinoma are expressing CD140α (**Figure 3C**).

Separation of T lymphocytes derived from human breast tumor

In order to understand how immune cells interact with breast tumor-derived stromal cells and cancer cells, we first isolated the T lymphocytes from the breast tumor microenvironment and expanded them in *ex vivo*. To remove the tumor infiltrating T cells (TILs) more effectively, we allowed TILs to migrate out from the tumor tissue naturally. This isolating method promotes viability and stability of TILs compared to conventional TIL cell separation technique by tissue digestion (enzymatic digesting using collagenase and liberase). Upon receiving a primary human breast tumor sample, the tissue was immediately dissociated into fragments (approximately ≤ 1 mm diameter) via mechanical dissociation and plated directly onto 0.1% gelatin coated culture flask containing RPMI media supplemented with 10% fetal bovine serum and rhIL-2 (50 ng/ml). Within 2 to 3 days in culture, we observed TILs migrating out from the breast tumor fragments. Shown in **Figure 4** is phenotype analyses of the TILs recovered from the culture. These results provide a population of TILs which exhibit both CD45RA (naïve) and CD45RO (memory) populations which may be utilized for further analysis.

Breast cancer secreted molecules on DC clustering and maturation using proteomics and gene expression analysis

In order to better evaluate the molecules or factors that may be affecting DC clustering and maturation, we must be able to re-create a suitable method for recapitulating the tumor microenvironment in a controlled setting. The 3D culturing techniques presented and used identify a more physiological method (versus monoculture *in vitro*) for identifying the interactions between tumor microenvironment and immune cells. The method of using 3D aggregate cultures will allow us to create an isolated environment of PBMC derived DCs along with tumor associated stroma or healthy stroma cells, and with or without primary tumor involvement if desired. The cells then can be analyzed via histological analysis, as demonstrated previously, from paraffin embedded 3D aggregate cultures to identify DC populations. Laser capture microdissection can then be utilized on these 3D aggregate sections and run for analysis of genetic expression changes of the DCs exposed to tumor associated stroma versus healthy stroma. Futhermore, results can be compared to genetic expression microarray profiles available in public domain. Specifically, utilizing a deconvolution methodology, which was developed by our lab (*10*), we are able to perform analysis of cell-type specific gene expression using existing large pools of publically available microarray datasets and identify if the trends found in our 3D aggregate analysis for DC populations is identified in other tumors/cancers (*10*). We also have begun banking tumor associated stromal RNA, healthy stromal RNA, as well as tumor RNA for future analysis on microarray or RNAseq to identify unique markers which may be influencing DC clustering or maturation as well as other potential implications to surrounding immune cells in the tumor microenvironment.

- **Task 2. Testing novel strategies to enhance DC clustering *in vivo*: months 1-48.**
  - **a.** Test agents known to modulate DC clustering and maturation in vitro (month 1-24).
  - **b.** Test molecules that block factors identified in task 1 (month 12-48).

The maturation status of dendritic cells (DCs) plays an important role in determining the nature of an immunologic response. Mature DCs are capable of eliciting an effective “immunogenic” response. An effective

anti-tumor response is elicited when DCs present tumor antigens to T cells leading to activation and proliferation of specific T cells. Over the past decade, there has been considerable effort made to characterize DC populations in cancer. A pivotal location to examine such immune-tumor interactions is the tumor-draining lymph node (TDLN). It is the site where tumor antigens are typically first presented to the immune system and a critical initial decision between immune activation and tolerance is made. We have previously shown that DCs in healthy lymph nodes (HLNs) tend to aggregate in large clusters of mature cells, whereas DCs in TDLNs tend to remain either un-clustered or form smaller clusters with fewer mature cells. This clustering behavior promotes functionality of DCs by increasing the number of proximal T cells compared to un-clustered DCs. Intriguingly, the degree of DC clustering within TDLNs correlated strongly with better clinical outcome in breast cancer patients.

Developing an *in vitro/ex vivo* 3D system to understand the mechanisms behind DC clustering:

To develop a system that will allow growing of DCs in lymph node (LN) like microenvironment, specific culture conditions that support the separate ex vivo growth of LN stroma was established. Breast cancer LN tissue from patients was mechanically dissociated into tiny tumor fragments (approximately ≤ 1 mm diameter) and plated directly onto gelatin coated culture flasks in medium containing recombinant human EGF (100 ng/ml). Similar to our earlier observation in tumor-associated stroma expansion in culture, monolayers of stroma were generated from LN tissue fragment. Phenotype characterizations of the monolayers generated in LN stroma cultures done after 3 weeks, demonstrate the LN stromal cells express similar surface molecules as primary breast tumor-associated stromal cells (**Figure 5A**). Similar to how we developed our tumor aggregate culture system, we generated a cell aggregate mixture of healthy human peripheral blood-derived DCs and human thymic stromal cells. The main purpose of this assay system is to evaluate whether CD11C+ CD14- DCs can aggregate in large clusters as we observed HLNs. Shown in **Figure 5B** is a tissue section generated from the DC+thymic stroma aggregate, stained with CD11C and CD1A (for immature DC marker). As expected, we observed the large population of DC cluster, demonstrating the efficacy of our 3D cell aggregate to study abnormal DC clustering in the LNs of cancer patients. Applying our current 3D cell aggregate system, the mechanisms behind DC clustering in both normal and cancer associated LN compartment will be evaluated.

Plans for the next 12 months:

- Integration of multiple cell types involved in *in vitro* 3D tumor microenvironment, especially tumor associated stromal cells, to determine factors which affect tumor progression and metastasis.

- Attempt to identify potential markers on stromal cells or cancer cells which may be producing unique immunosuppressive properties.

- Perform analysis on tumor associated and healthy stroma in the tumor microenvironment by microarray or RNAseq to help identify unique genetic expression profiles or functional defects which may influence tumor progression/metastasis.

- Test possible agents which may modulate immunosuppressive conditions in 3D microenvironment and expand 3D culture aggregate system with use of 3D hydrogel matrix.

- Monitor effect of modulation of DC clustering/maturation by testing known agents previously investigated and mentioned above, by utilizing assays in 3D aggregate cultures and 3D hydrogel matrix systems.

- Once candidate agents/therapeutic markers for cancer-stroma or DC functionality are proven *in vitro* via IHC or FACS based assays, we will expand to analyze effects *in vivo* murine models to identify effects in TDLN.

Supporting Data/Figures:

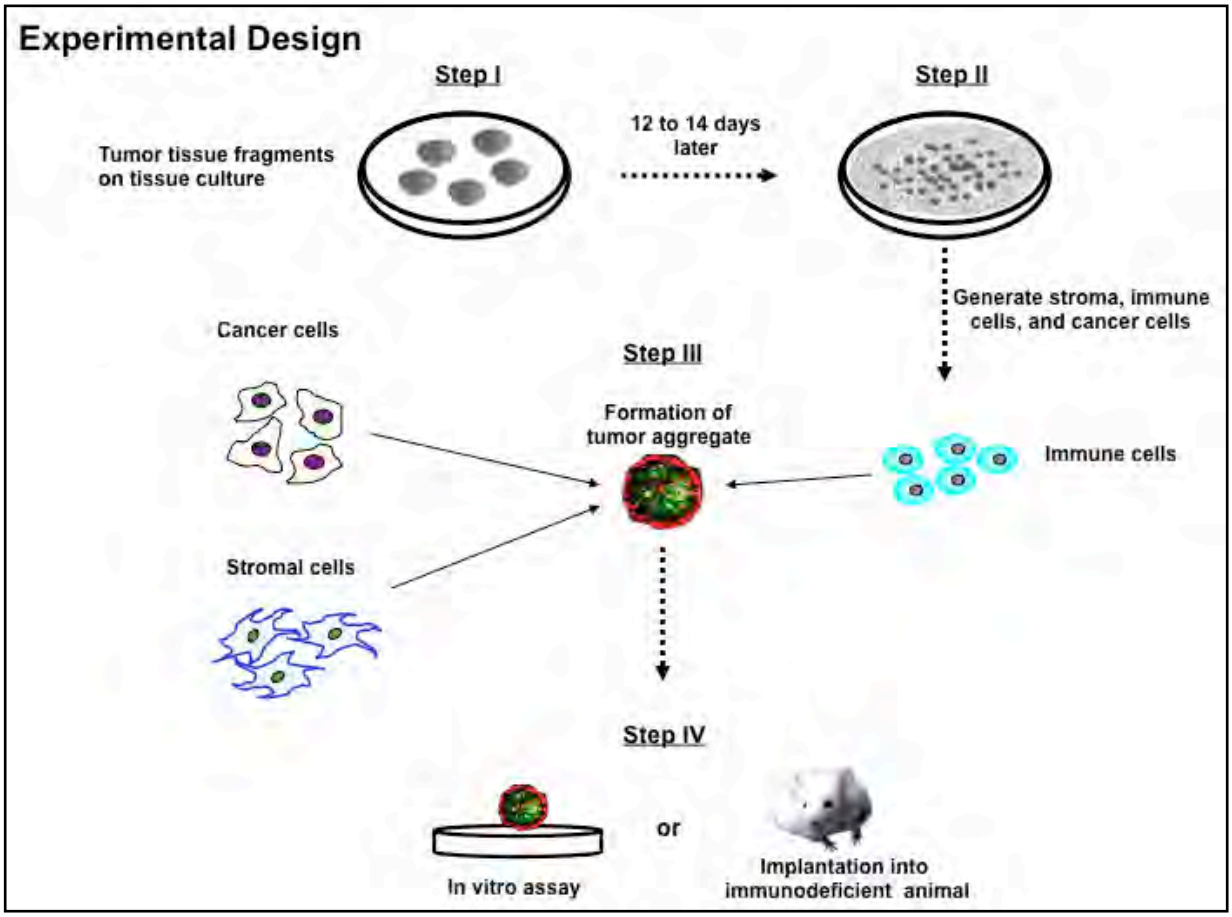


Figure 1- Experimental Design: Illustration of the steps involved in producing 3D aggregate populations from tumor associated cells and use *in vitro* or potentially *in vivo.*

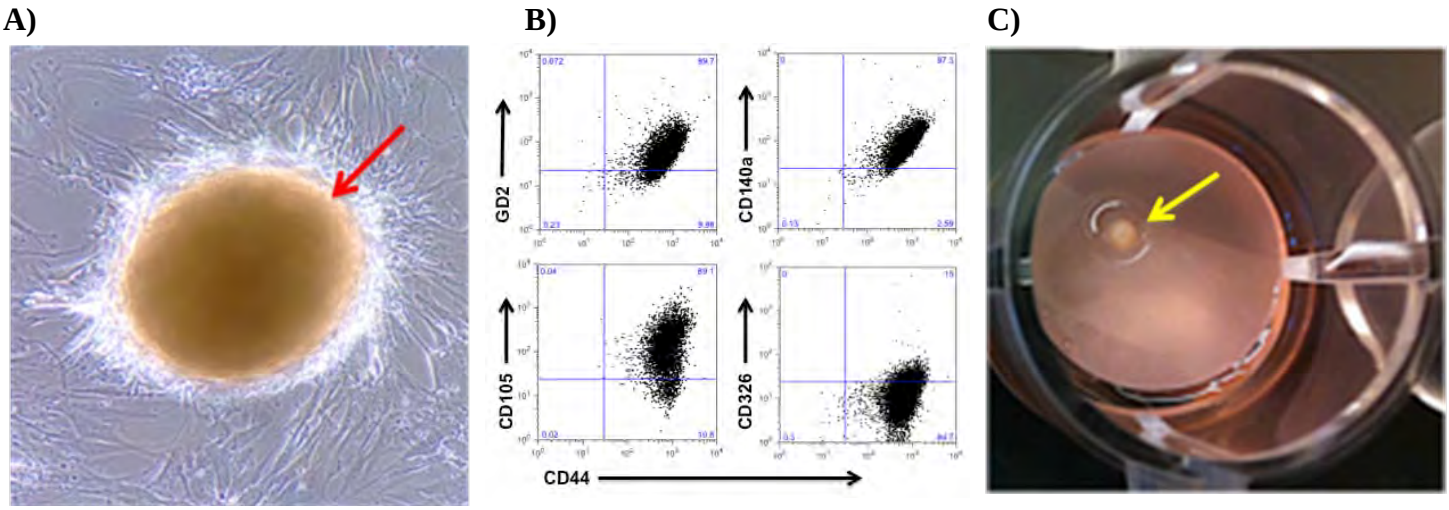


Figure 2: A) Morphology of stromal cells generated in culture, 3 weeks after initiation from human breast tumor fragments (red arrow). **B**) At 3 weeks, cell surface marker expression was analyzed by FACS on monolayers formed in stroma tissue culture condition. **C**) Cultured human breast tumor-derived storal cells were spun down to form aggregates. Aggregates were then cultured on nucleopore membranes floating in DMEM medium supplemented with 10% FBS for 5-7 days for histological staining analyses.

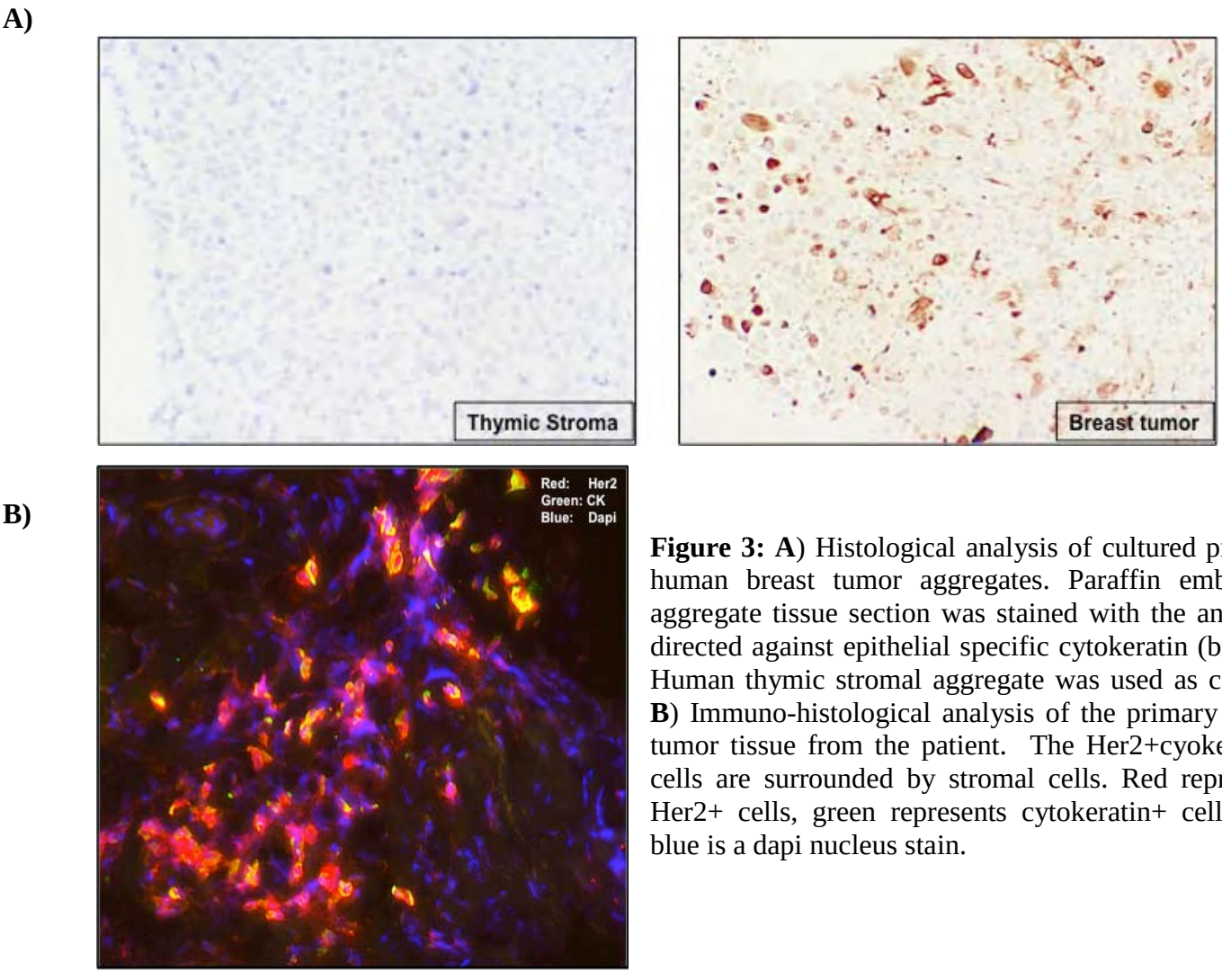


Figure 3: A) Histological analysis of cultured primary human breast tumor aggregates. Paraffin embedded aggregate tissue section was stained with the antibody directed against epithelial specific cytokeratin (brown). Human thymic stromal aggregate was used as control. **B**) Immuno-histological analysis of the primary breast tumor tissue from the patient. The Her2+cyokeratin+ cells are surrounded by stromal cells. Red represents Her2+ cells, green represents cytokeratin+ cells, and blue is a dapi nucleus stain.

C)

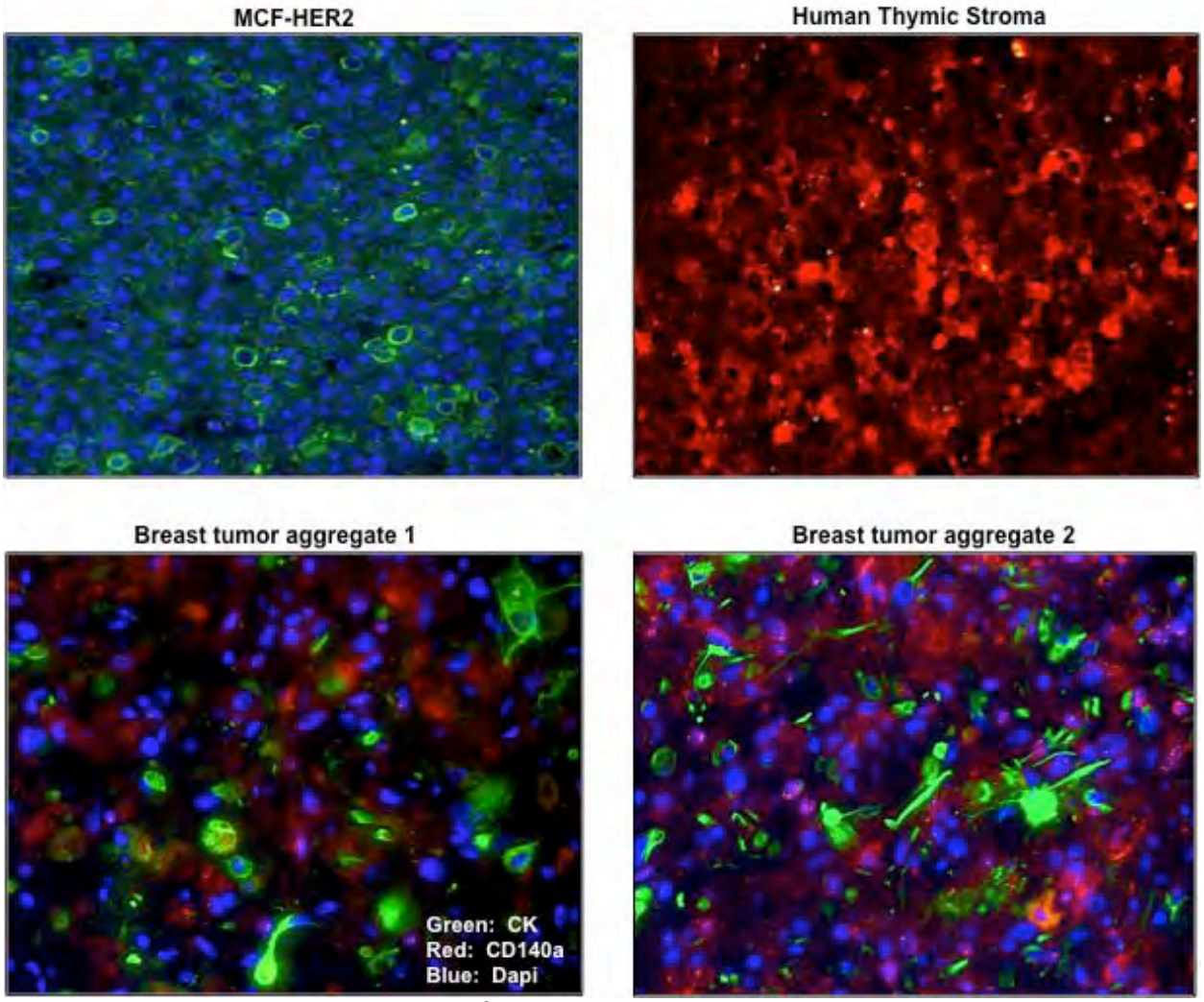


Figure 3: **C**) Immuno-histological analysis of the primary breast tumor aggregates in culture. Aggregates were cultured for 7 days, and paraffin embedded aggregate sections were stained with the antibodies directed against CD140α and cytokeratin. MCF-Her2 human breast cancer cell line and human thymic stromal cells were used as both negative and positive controls.

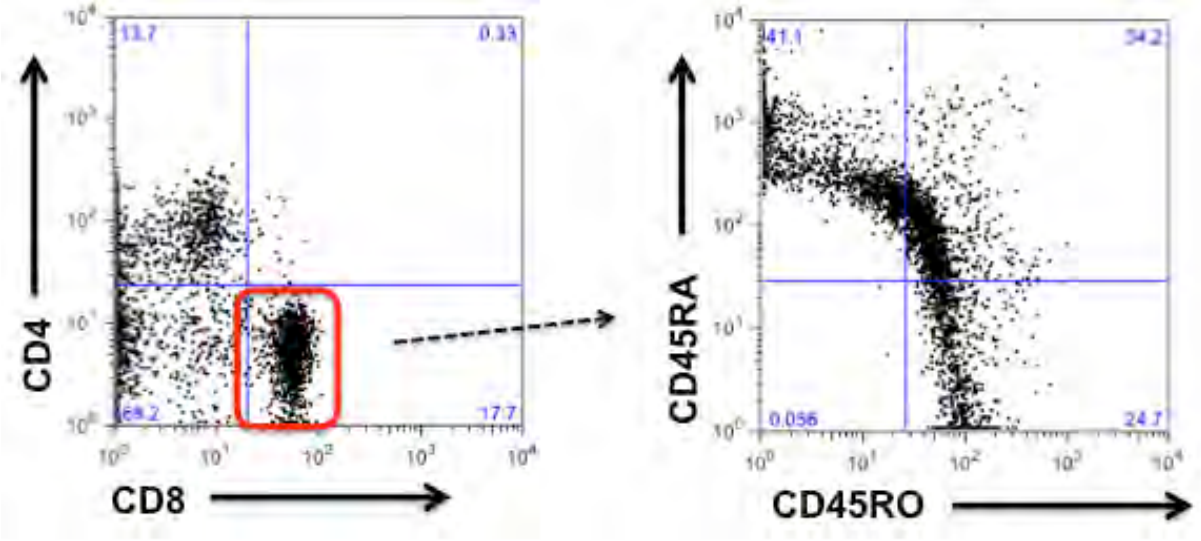


Figure 4: FACS analysis of TILs from the primary breast tumor tissue. CD8+ CTLs were gated and analyzed for the expression of CD45RA (naïve) and CD45RO (memory).

A)

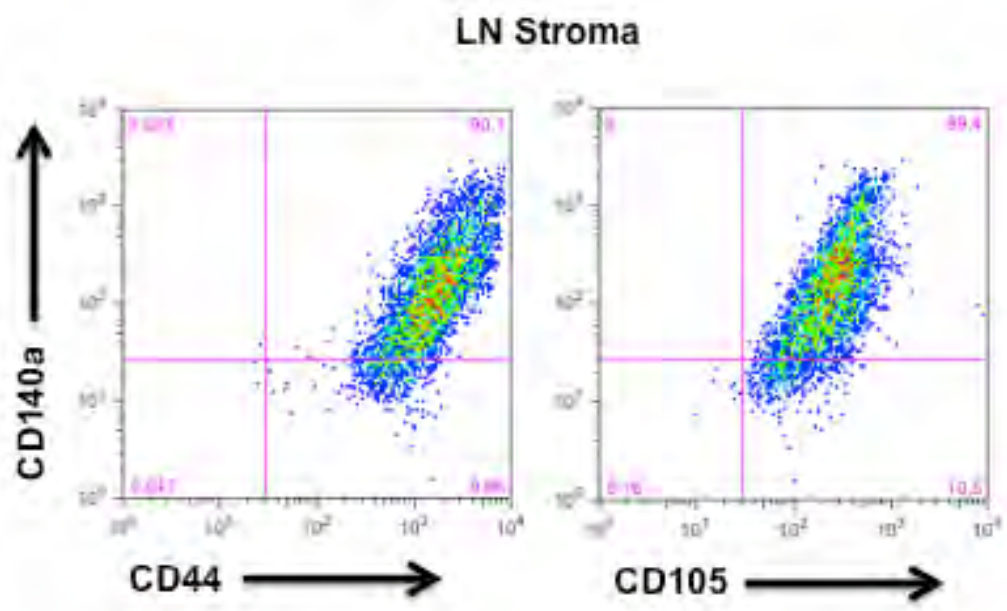


Figure 5: A) Phenotypic characterization of LN-derived stromal cells following ex-vivo expansion. These stromal cells are also negative for CD45 and CD11C.

B)

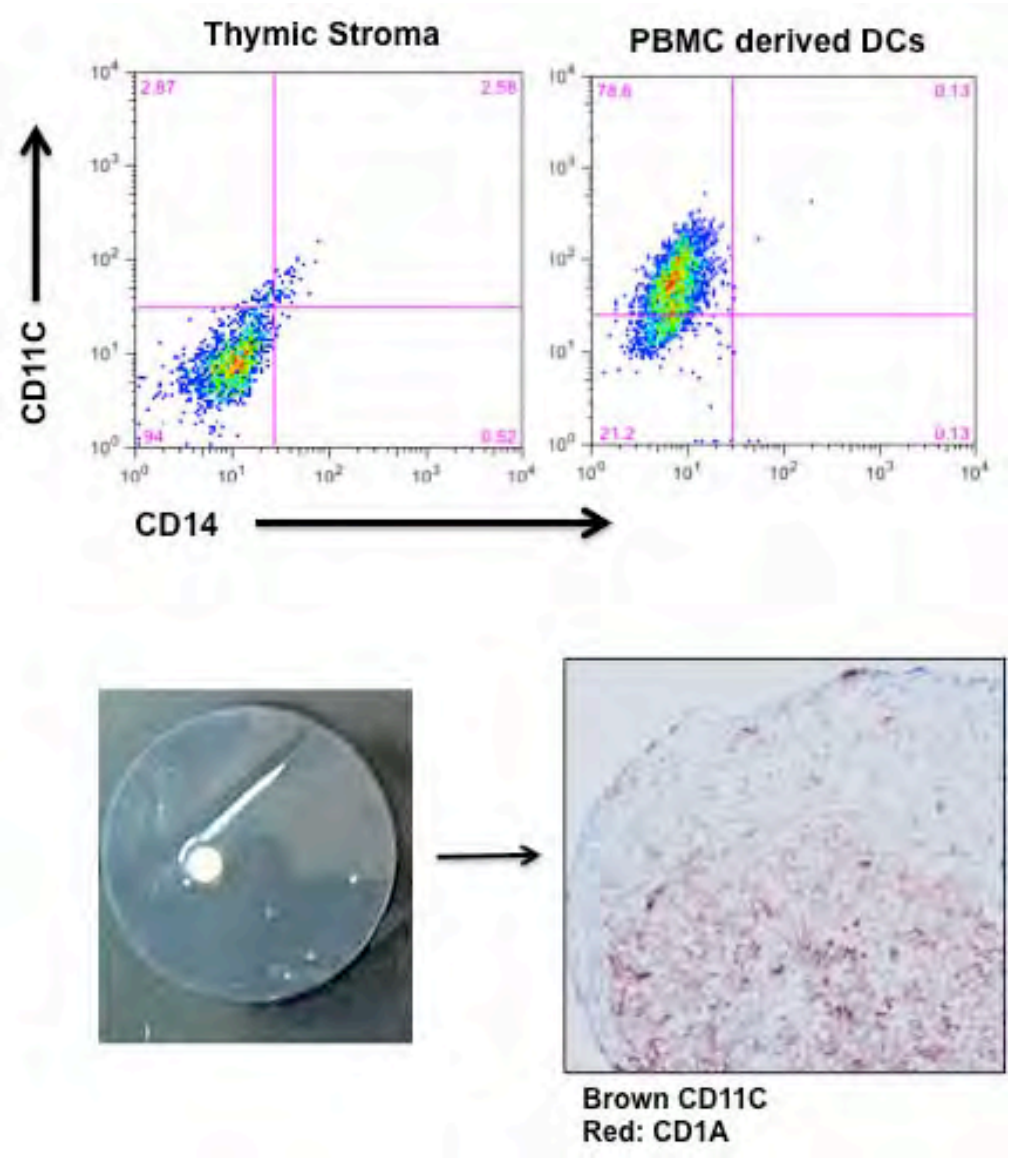


Figure 5: B) Healthy peripheral blood derived DCs were generated from mono-nuclear cells cultured with GM-CSF (50 ng/ml) and IL-4 (100 ng/ml) for 10 days. After 10 days, these cells were analyzed for their DC surface markers via FACS. Most of these DCs express CD11C and CD86 but negative for CD14. In comparison, human thymic stromal cells are negative for CD11C. Both thymic stroma and DCs were mixed together (ratio of 5:1) as a cell aggregate and cultured for additional 7 days. The paraffin embedded section was stained with the antibodies directed against CD11C and CD1A.

<u>Aim 2. Enhance T cell function *in vivo* by restoring immune signaling</u>.

- **Task 3. Investigating mechanisms by which chronic IL-6 affects immune function: months 1-36.**
  a. Gene expression analyses and flow cytometry to measure expression of positive and negative signaling regulators (month 1-36).
  b. T cell polarization and functional assays (month 1-36).

Multiple immune defects have been documented in cancer patients, which limit the ability of the host immune response to control cancer progression and metastases to prevent relapse. In addition, tumor-promoting immune functions have been shown to be up-regulated in cancer patients. The universal dysfunction of the

immune system could be explained by a breakdown in communication via cytokines. IL-6 is a cytokine that signals through a receptor complex of GP130 and IL-6Ra to activate the STAT3 and STAT1 transcription factors (*11*). IL-6 has pleiotropic roles in disease and immune responses with a well-known role in promoting tumorigenesis. In cancer settings, IL-6 is produced by tumor cells, tumor stroma and tumor-associated myeloid cells and activates phosphorylation of STAT3 (pSTAT3) in tumor cells to promote survival and proliferation. STAT3 itself is considered an oncogene and also promotes the renewal of cancer stem cells (*12*).

Breast cancer patients are known to have elevated serum levels of IL-6, and higher serum IL-6 levels are associated with poorer survival in metastatic breast cancer patients (*13, 14*). Because of the strongly supported role of IL-6 in promoting tumorigenesis, multiple efforts are underway to inhibit IL-6 as a therapeutic intervention. For immune cells, IL-6 has vital roles in T cell activation, for instance by inhibiting T_{REG} while promoting T_H17 differentiation (*15*). Mice lacking IL-6 are unable to elicit effective immune responses against viruses and bacteria (*16, 17*). T cells from breast cancer patients are known to have impaired effector functions and are skewed towards T_{REG} populations. However the role of IL-6 in breast cancer patient T cell function has not been previously addressed. IL-27 also signals through GP130 paired with a unique receptor WSX-1 to activate STAT1 and STAT3 (*18*). IL-27 is known to enhance T_H1 and inhibit T_H2 polarization by synergizing with IL12 to promote IFNγ production and inhibiting T_H2 cytokine production (*19-21*). IL-27 was also shown to induce generation of effector CTLs from naive $CD8^+$ T cells in a STAT1-dependant manner (*22, 23*). Interestingly, IL-27 has anti-tumor activities *in vivo* in mice (*24*).

While both IL-6 and IL-27 activate STAT1 and STAT3, IL-6 tends to favor STAT3 activation, and IL-27 favors STAT1. STAT1 and STAT3 cross-regulate one another by competing for binding sites on cytokine receptors, upregulating expression of negative regulators, and can form heterodimers to alter promoter binding specificities (*25*). STAT1 and STAT3 mediate opposing effects on both tumor cell survival and immune cell activity. In tumor cells, STAT3 promotes survival and proliferation. In contrast, STAT1 promotes cell cycle arrest and apoptosis (*25*). In immune cells, cytokines, including IL-10 and VEGF, activate STAT3-mediated immune suppression, while STAT1 activation promotes antigen presentation, inflammatory responses, and T_H1 immunity (*25*). Immune cells lacking STAT3 exhibit enhanced tumor immune-surveillance (*26, 27*). These studies indicate the crucial balance of cytokine signaling in directing anti-tumor immune responses.

Regulation of IL-6 & IL-27 signaling, GP130 and IL-6Rα expression, and STAT1 and STAT3 expression levels by chronic IL-6 stimulation

PBMCs were pre-treated with increasing concentrations of IL-6 for 24 hours, and subsequently stimulated with either IL-6 or IL-27 to measure STAT1 & STAT3 responsiveness. Pretreatment with IL-6 dose-dependently down-regulated IL-6 and IL-27 activation of pSTAT1 and pSTAT3 in all IL-6-responsive T cell types (**Figure 6**). In contrast, pretreatment with IL-27 or IFNγ had the opposite effect and promoted pSTAT1 activation in response to IL-6 and IL-27, while also reducing basal levels of pSTAT3 (**Figure 6**). These results indicate IL-6 hypo-responsiveness by down-regulating GP130 and IL-6Rα, and furthermore extending defective responsiveness to other GP130-dependant cytokines including IL-27. This defect is also evident when looking at the regulation of GP130 and IL-6Rα expression by chronic IL-6 and IL-27 stimulation. Chronic stimulation with IL-6 decreased surface protein expression levels of GP130 and IL-6Rα in IL-6 responsive T cell subsets, while IL-27 treatment had the opposite effect (**Figure 7**). Furthermore, we observed a regulation of total STAT1 & STAT3 expression by chronic IL-6 stimulation. The results indicate that chronic stimulation with IL-6 decreased expression levels of total STAT3 in T cell subsets from healthy donor PBMCs but had no effect on expression levels of total STAT1 (**Figure 8**).

In total these results indicate that PBMCs from healthy donors chronically treated with IL-6 resulted in significant downregulation of GP130 expression levels in CD4 naive, CD4 memory, and CD8 naive T cells, but not in IL-6-nonresponsive (IL-6Rα-negative) CD8 memory T cells. In addition, IL-6Rα expression levels were also downregulated in CD4 naive, CD4 memory, and CD8 naive T cell subsets following chronic IL-6 treatment. Thus, chronic exposure of T cells to IL-6 results in a loss of GP130 and IL-6Rα expression in IL-6 responsive T cell subsets and may resemble the reduced levels of these receptors observed in cancer patients. With regards to STAT1 and STAT3 signaling, our results attempt to identify the unique role of STAT3 and

STAT1 in immune regulation. We do see that chronic IL-6 or IL-27 exposure may down regulate pSTAT3 and pSTAT1 expression but only a reduction in total STAT3 versus total STAT1 in chronic IL-6 conditions. These results underscore the importance to understand the roles of STAT3 and STAT1 in the immune cell population in the context of cancer and decipher their global impact. These data provide the groundwork to explore modulating IL-6 or IL-27 cytokines (which play integral role in immune signaling and tumor progression) in search for beneficial immunotherapeutic options against multiple cancer types.

Regulation of T cell survival and proliferation by IL-6 under TCR and/or PHA stimulated conditions

To determine the potential for T cells to survive and proliferate in conditions were there was physiologically healthy level or increasing level of IL-6 present in the microenvironment, we ran assays to monitor the T cell survival as well as the proliferation potential in the presence of IL-6 when stimulated by either TCR or PHA. Our preliminary results show that when culturing of PBMCs in the presence of IL-6, there was an enhanced survival of the PBMCs and would point towards an enhanced T cell population considering the TCR-stimulated conditions (**Figure 9**). Thus, if proper IL-6 signaling can be restored in cancer patients, then they might be able to more efficiently mount an immune response. To further illustrate the importance of IL-6 on PBMCs, and specifically T cell populations, we performed assays to identify proliferation of these groups. Culturing of PBMCs in the presence of IL-6 enhanced proliferation under TCR or PHA stimulated conditions, specifically of CD4 and CD8 T cell populations (**Figure 10**). In relation to potential immunotherapeutic potential, it is of great benefit to have a viable and healthy T cell population present at the tumor or in the tumor microenvironment to promote a positive patient outcome. This work provides a very important groundwork to how to better design a combined immunotherapeutic option by identifying the unique roles of these immune signaling events in relation to cancer as we move forward.

- **Task 4. Testing the effectiveness of IL-6-blockade plus IL-27 treatment in reversing chronic IL-6-induced T cell dysfunction: months 12-48.**
  a. In vitro assays to test the effects of treatment with an anti-IL-6 antibody plus IL-27 during naive CD4 T cell polarization and CD8+ T cell activation (month 12-36).
  b. Test various treatment strategies to reverse T cell dysfunction in BC patients (month 24-48).

Our initial goal was to measure and assess the effect of treatment with an anti-IL-6 antibody plus IL-27 during naive CD4 T cell polarization and $CD8^+$ T cell activation. The data will increase our understanding of how immune signaling defects occur and affect T cell functions in breast cancer patients and how cytokines such as IL-27 or IFNɣ could be therapeutically administered or inhibited to correct immune dysfunction. To determine the ability of these treatment strategies to reverse T cell dysfunction in breast cancer patients, PBMCs from breast cancer patients and healthy controls were treated with an anti-IL-6 antibody followed by assessment of GP130 and IL-6Rα receptor as well as STAT1 and STAT3 expression and also IL-6 and IL-27 signaling defects in healthy donor PBMCs. Further analysis then could be made on the unique effects on CD4 and CD8 T cell populations, and thus an assessment of the anti-IL-6 treatment may be beneficial for either anti-tumor responses or pro-immune activity against cancer.

Our hope is that inhibiting IL-6 and administering IL-27 may be an ideal combination—along with other immune modulating therapies such as DC vaccination—to promote the optimal environment for generation of productive anti-tumor T_H1 and maintaining functional CTL responses *in vivo*.

Regulation of GP130 and IL-6Rα expression, STAT1 and STAT3 expression, and IL-6 & IL-27 signaling by anti-IL-6 treatment.

To help identify methods of reversing potential defects of immune cells or immunosuppressive environments via cytokine regulation, we investigated the regulation of GP130 and IL-6Rα expression by anti-IL-6 treatment. The experimental approach was again, to mimic a tumor microenvironment by pre-exposing PBMCs, with chronic stimulation of IL-6. This would result in a hypo-responsiveness to IL-6 as demonstrated

in previous work. In addition, we also pre-exposed PBMCs, in a separate experiment, with an anti-IL-6 antibody to determine if the blockade would restore expression of GP130 and IL-6Rα. Results seen in **Figure 11** indicate that there is a lower expression of both GP130 and IL-6Rα mainly in CD4 T cell subsets when pre-treated with 10ng/ml IL-6. Whereas, there is a higher expression of GP130 and IL-6Rα, again in CD4 T cell population, when pre-treating with anti-IL-6 at 5ug/ml. These results may be a positive indication that receptor expression may be restored with the anti-IL-6 and potentially useful to restore IL-6 responsiveness in immune cell populations.

The expression of total STAT1 and STAT3 in the presence of anti-IL-6 treatment was also analyzed. Results show that there is no effect with anti-IL-6 treatment in on expression of total STAT3 and STAT1. Looking at the distinct T cell populations (**Figure 12**), it appears that anti-IL-6 treatment of PBMCs has no significant effect the total STAT3 or STAT1 expression in both CD4 and CD8 naïve and memory subsets with anti-IL-6 treatment.

Finally, we also were able to provide results regarding the changes of IL-6 & IL-27 signaling in the presence of anti-IL-6 treatment with respect to pSTAT1 and pSTAT3. PBMCs from healthy donors were pre-treated with chronic IL-6 or anti-IL-6 antibody as previously described. The results show that subsequent IL-6 and IL-27 stimulation show enhanced pSTAT1 and pSTAT3 responses when pre-treated with anti-IL-6 in both CD4 and CD8 naïve and memory T cells, but a more pronounced recovery of signaling for both IL-6 and IL-27 was exhibited in the CD4 subset. As for the pSTAT3 response to stimulation with IL-6 or IL-27, we observed the same enhanced pSTAT1 and pSTAT3 signaling in the presence of anti-IL-6, but again, more pronounced in the CD4 T cell subset. The opposite effect was found with treatment of PBMCs with chronic IL-6 (**Figure 13**). These results are an exciting avenue for being able to overcome hypo-responsiveness of IL-6 signaling and restore proper immune function by utilizing blockade of IL-6. This will be explored more in depth as we move forward and hopefully expand to determine effects *in vivo*.

Plans for the next 12 months:

- Potential agents which may enhance or restore cytokine balance (IL-6 / IL-27 responsiveness) will be analyzed in 3D aggregate culture. Including investigation of stromal cell impact on cytokine signaling consequences on multiple cell types in the tumor microenvironment.

- Continued gene expression data to be collected in specific tumor conditions to identify the potential impact cytokine regulation has on multiple types of T cells and their ability to function as well as potential DC clustering/maturation impact.

- IL-6 blockade will be further assessed *in vitro* so that *in vivo* analysis can be carried out appropriately.

- Identification of proper methods of restoring CD4 and CD8 T cells will be studied as well, utilizing cytokine blockade *in vitro*, before moving findings to *in vivo* model.

- Determine if other factors such as immune aging, may be adversely affecting the cytokine signaling and subsequently complicating immune anti-cancer responses.

Supporting Data/Figures:

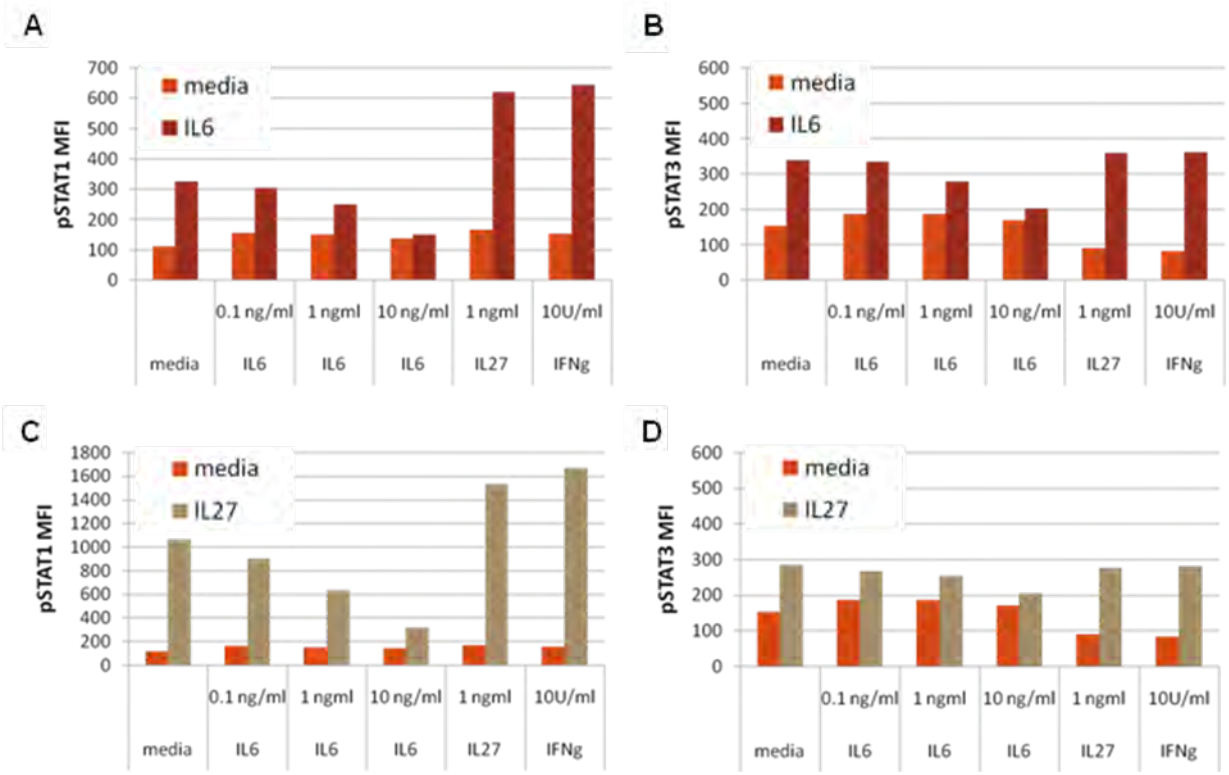


Figure 6. Negative Regulation of IL-6 & IL-27 signaling by chronic IL-6 stimulation. Healthy donor PBMCs were pretreated for 24 hours with indicated doses of IL-6, IL-27, IFNg, or untreated (media) as indicated by labels on the X axis. After 24 hours, cells were stimulated with 100ng/ml IL-6 (**A, B**), 50ng/ml IL-27 (**C, D**), or unstimulated (media). pSTAT1 (Y701) (**A, C**) and pSTAT3 (Y705) (**B, D**) were measured by phospho-flow cytometry in CD4 T cells. Similar results were obtained for other T cell subtypes.

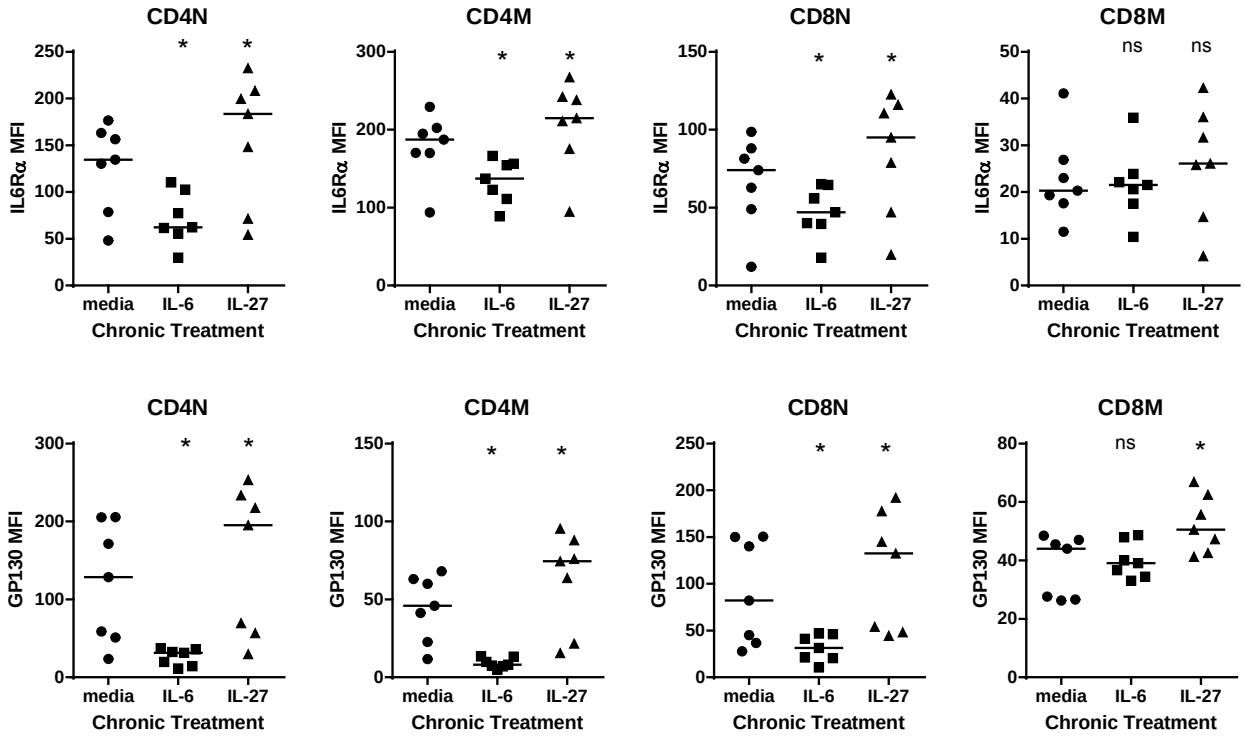


Figure 7. Regulation of GP130 and IL-6Ra expression by chronic IL-6 and IL-27 stimulation. Healthy donor PBMCs were pretreated for 36 hours with 10ng/ml IL-6. After 36 hours, naïve and memory CD4 and CD8 T cells were analyzed by flow cytometry for surface expression of IL-6Ra and GP130. Values represent Median Fluorescence Intensity (MFI) of the isotype control subtracted from the specific signal. P-values were determined by paired Mann-Whitney tests. * = $p < 0.05$; ns = not significant.

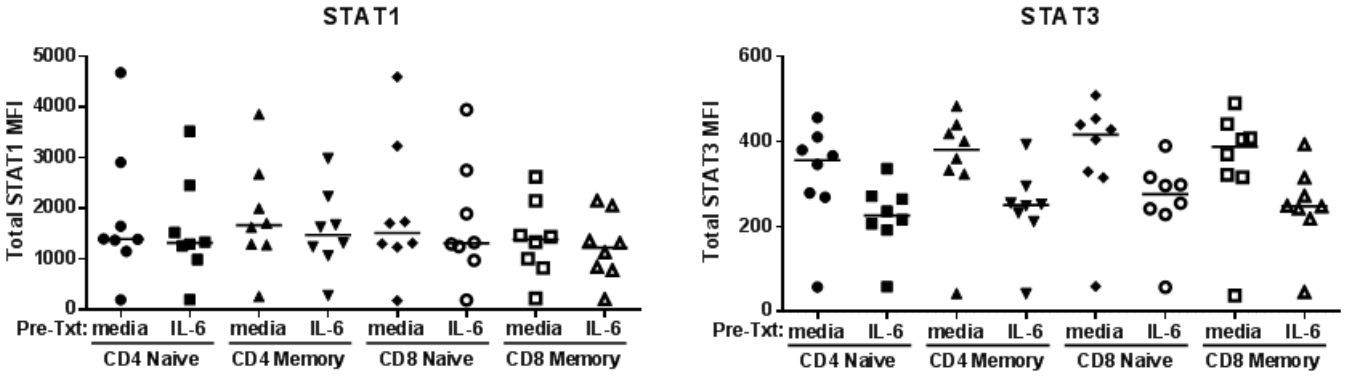


Figure 8. Regulation of STAT1 & STAT3 expression by chronic IL-6 stimulation. Healthy donor PBMCs were pretreated for 36 hours with 10ng/ml IL-6. After 36 hours, naïve and memory CD4 and CD8 T cells were analyzed by flow cytometry for surface expression of total STAT1 and STAT3. Values represent Median Fluorescence Intensity (MFI) of the isotype control subtracted from the specific signal.

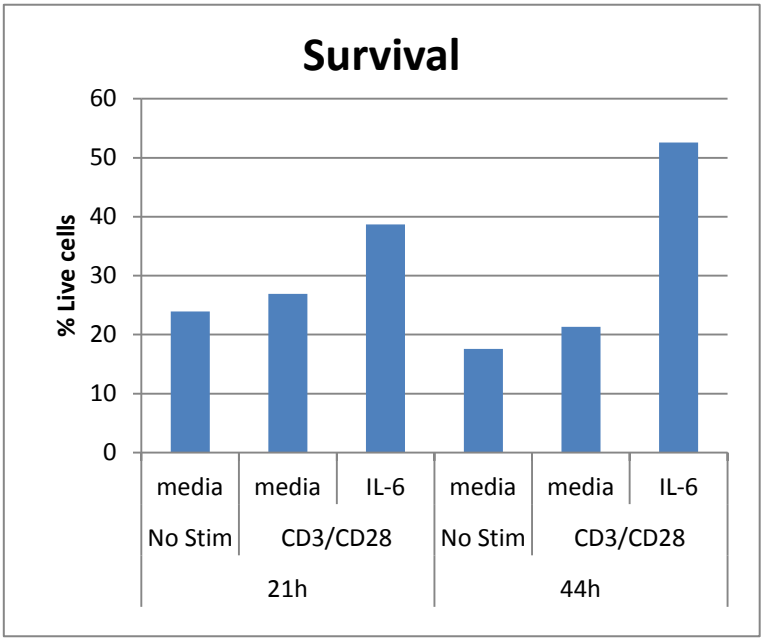


Figure 9. Regulation of survival by IL-6. Healthy donor PBMCs were unstimulated or cultured with plate-bound anti-CD3 + anti-CD28 antibodies, with or without 10ng/ml IL-6, for 21 or 44 hours. PBMCs were analyzed by flow cytometry for Annexin V and Live-Dead staining. The frequency of Annexin V$^-$Live-Dead$^-$ cells for each treatment is indicated.

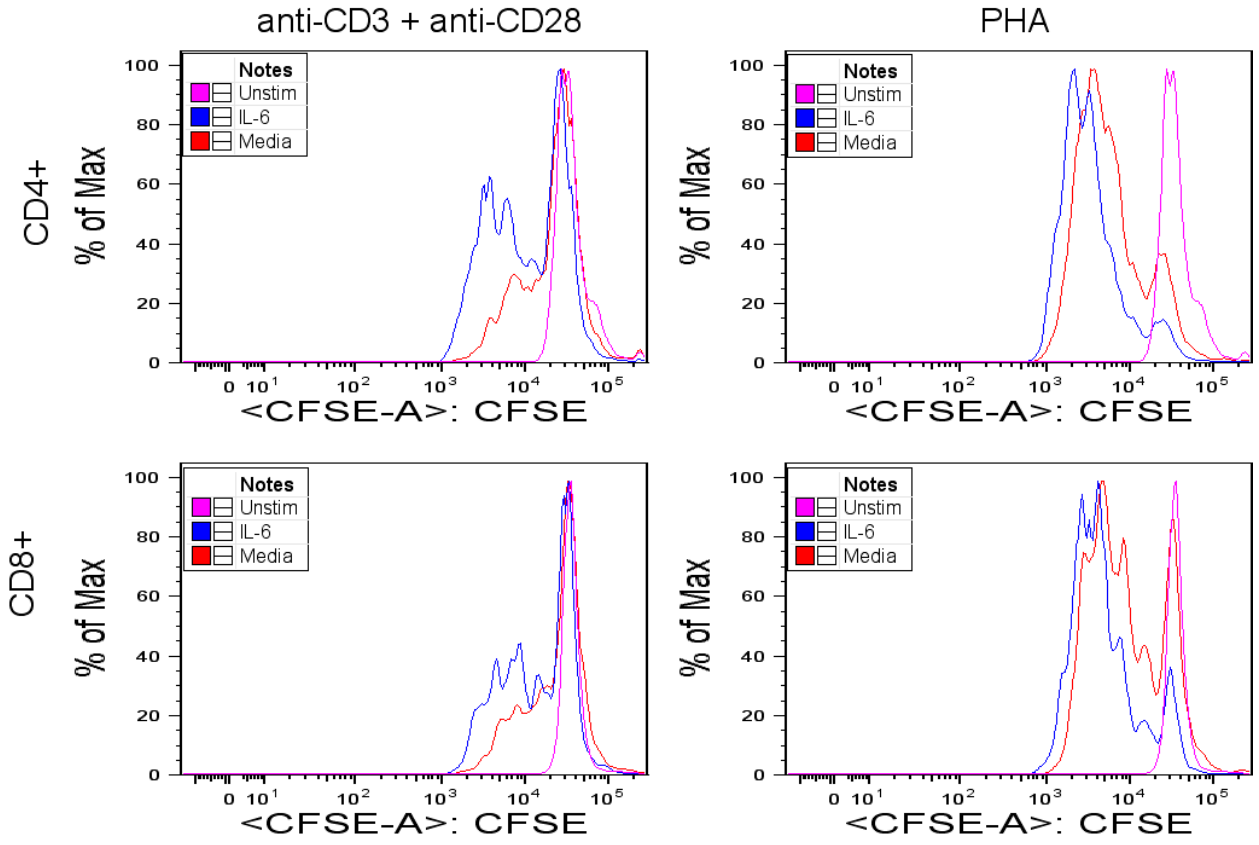


Figure 10. IL-6 enhances TCR and PHA driven T cell proliferation. Healthy donor PBMCs were labeled with CFSE and unstimulated or cultured with plate-bound anti-CD3 + anti-CD28 antibodies or PHA, with or without 10ng/ml IL-6, for 4 days. PBMCs were analyzed by flow cytometry for CFSE staining. CFSE staining in CD4$^+$ and CD8$^+$ T cell populations is shown.

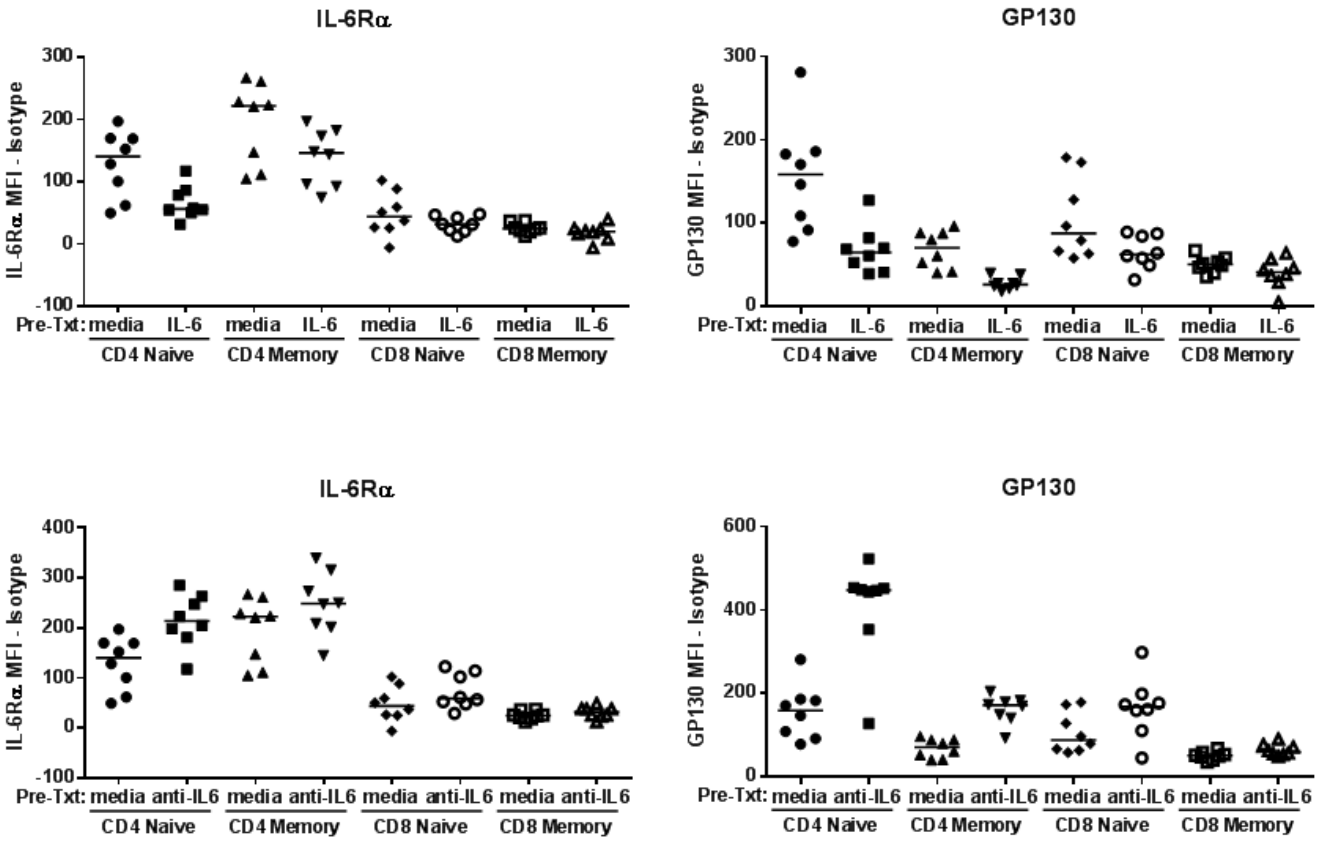


Figure 11. Regulation of GP130 and IL-6Ra expression by chronic IL-6 stimulation. Healthy donor PBMCs were pretreated for 36 hours with 10ng/ml IL-6 or anti-IL-6 antibody at 5ug/ml. After 36 hours, naïve and memory CD4 and CD8 T cells were analyzed by flow cytometry for surface expression of GP130 and IL-6Ra . Values represent Median Fluorescence Intensity (MFI) of the isotype control subtracted from the specific signal.

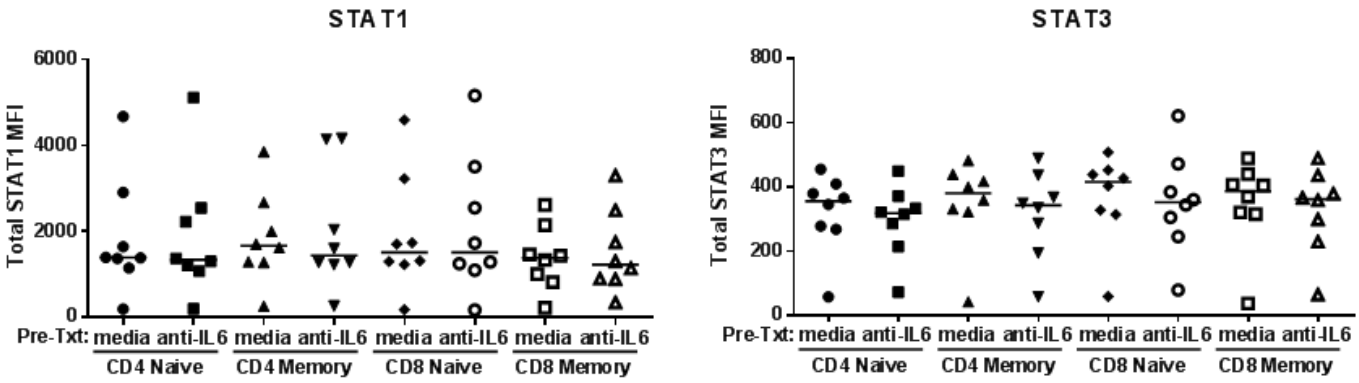


Figure 12. Regulation of STAT1 & STAT3 expression by inhibiting IL-6 stimulation. Healthy donor PBMCs were pretreated for 36 hours with anti-IL-6 antibody at 5ug/ml. After 36 hours, naïve and memory CD4 and CD8 T cells were analyzed by flow cytometry for surface expression of total STAT1 and STAT3. Values represent Median Fluorescence Intensity (MFI) of the isotype control subtracted from the specific signal.

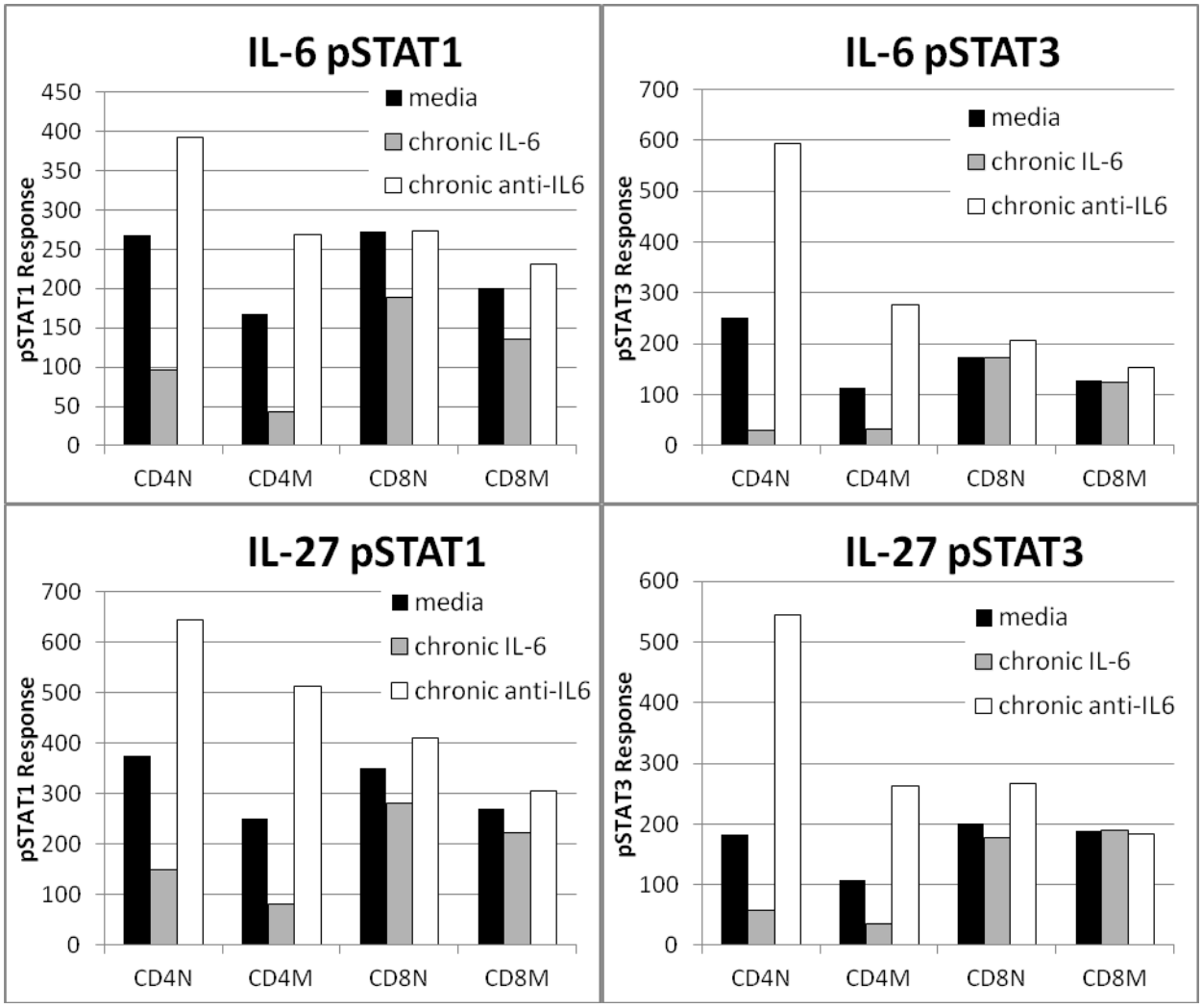


Figure 13. Enhancement of IL-6 & IL-27 signaling by anti-IL-6 antibody. Healthy donor PBMCs were pretreated for 36 hours with 10ng/ml IL-6, 5ug/ml anti-IL-6 antibody, or untreated (media) as indicated by legend. After 36 hours, cells were stimulated with 100ng/ml IL-6 or 50ng/ml IL-27, or unstimulated (media). pSTAT1 and pSTAT3 responses determined by subtracting unstimulated MFI from IL-6 or IL-27-induced MFI for the indicated cell types.

Aim 3. Select optimal integrated immunotherapy combinations in animals for clinical development.

- **Task 5. Select Optimal Integrated Immunotherapy Combinations in Animal Models for Clinical Development: months 12-60.**

While some combinations of FDA approved cytokine therapeutics for cancer (IL2 and IFNα2b) have shown modest incremental efficacy, they have been limited by substantial toxicities (28). Previous cytokines tested clinically as cancer therapeutics were selected based on putative effects to enhance immune function rather than specifically to correct immune signaling defects in cancer. In addition, DC vaccination effectiveness may be hampered by an immunosuppressive environment, which prevents maturation and clustering in lymph nodes. Each of the key phases of the immune response—induction, amplification, and effector immune cell generation—is defective in breast cancer patients, so it follows that integrated immunotherapy combinations that address all of the phases of the normal immune response will be much more effective than individual treatments that address only one mechanism.

Developing integrated immunotherapeutic regimens to target the immune signaling defects that occur in each phase of the immune response (**Figure 14**), in combination with optimal DC-vaccination strategies using immunologically validated antigens, will generate highly functional and prolonged anti-tumor immune responses in breast cancer patients that will prevent recurrence and metastasis.

We will focus on developing a thorough approach to best take our findings about the 3D tumor microenvironment and its unique setting with our knowledge of cytokine signaling functions in immune and cancer cells and then attempt to assess *in vivo* effectiveness of combining the regimens for induction of optimal anti-tumor immunity. Then we will determine the optimal time to administer these regimens during disease progression, with and without chemotherapy. As surgery removes the primary tumor burden, we will focus our studies on the post-surgical setting where we envision immunotherapy is most effective to eradicate micrometastases to prevent relapse.

Task 5a. Optimize post-surgical murine model of breast cancer metastasis (month 12-36).

To identify optimal integrated cancer immunotherapy approaches and evaluate their efficacy in murine models of breast cancer, we decided to take advantage of the well characterized and widely used murine 4T1 Balb/c ER/PR/Her2 triple-negative breast cancer model. The newly derived 4T1.2 breast cancer cell line has demonstrated superior metastatic potential and disease progression closely mimicking human disease. 4T1.2 cells were obtained from Robin Anderson, Peter MacCallum Cancer Research Centre, Australia. As an alternative transplantable Her-2-positive model of breast cancer we considered the use of the DDHer2 cell line, derived from a spontaneous breast tumor in Balb/c Her-2/neu transgenic mice. The potential need to study the effect of different combinatorial cancer immunotherapy strategies on anti-tumor antigen-specific responses prompted us to explore the possibility of using engineered OVA-expressing B16 melanoma or E0771 breast cancer cells. These are two highly metastatic tumors on the C57BL/6 background that can be combined with H-2b-restricted Ova peptides as well as adoptive transfer of Ova-specific OT-I/OT-II TCR transgenic T cells.

4T1, 4T1.2, E0771 and B16 cells were transfected with retroviral vectors to stably express GFP and luciferase, which was documented by flow cytometry analysis and luminescent analysis (**Figures 15 and 16**). Gilham et al. modified the retroviral vector rKat.43.267bn (Cell Genesys) by the inclusion of an Internal Ribsome Entry Site (IRES) enhanced green fluorescence protein (EGFP) element from the vector pIRES2.EGFP (Clontech) to form rKat.IRES.EGFP, then further modified this by insertion of firefly luciferase (Luc2) upstream of IRES.GFP. The single genome pkat2ampac retroviral packaging plasmid was constructed from pSKII (Stratagene) by insertion of the hCMV IE and MMLV U3 enhancer-promoter, the splice acceptor of human α globin gene exon 2, the SV40 early region polyadenylation site and SV40 origin of replication, the gag-pol genes from ecotropic MMLV, and the envelope gene from the 4070A amphotropic MLV .Our plan was to use GFP/Luc-expressing tumor cells in combination with total body biophotonic imaging to detect tumor cell growth *in vivo* as well as to quickly evaluate responses to various therapeutic combinations.

The priming of potent anti-tumor responses is dependent on the release of tumor-associated antigens as well as on the activation and functional status of the immune cells, including T cells and antigen presenting cells. Surgical removal of the tumor eliminates the natural reservoir of tumor antigens and can be supplanted with various strategies to induce immunogenic cell death *in vivo* resulting in spontaneous endogenous vaccination effect. Certain chemotherapeutic like doxorubicin have been reported to promote secondary anti-tumor immune mediated effects. High dose chemotherapy, on the other hand, is immunosuppressive and is not compatible with the goals of integrated cancer immunotherapy (**Figure 14**). Identifying highly synergistic combination of drugs and lowering the necessary dose of chemotherapeutic agents is therefore a promising alternative approach.

Using gene expression data from the MCF7 human breast cancer line we have identified several combinations of chemotherapeutic (doxorubicin, mitomycin C, paclitaxel) and secondary drugs (Naringenin, Adiphenine, NS-398, Ivermectin, Ascorbic Acid) predicted to have highly synergistic anti-tumor effects. We attempted to validate these predictions using the murine 4T1.2 breast cancer cell line, which we intend to use for subsequent *in vivo* mouse studies, as well as in the human MCF7 cells, that were used as a positive control. Synergy was evaluated using the CytoTox 96 assay based on the release of LDH from dying or detergent-lysed live tumor cells (**Figure 17**).

Several promising synergistic effects were observed, and the results are summarized in **Figure 18**. Notably, some of the secondary drugs had direct anti-tumor effects, while at the same time being minimally toxic to normal human PBMCs. Some of the secondary drugs also promoted unexpected expansion of NK cells, an effect that needs to be explored in further studies (**Figure 19**). The early activation and cytokine production from innate immune cells such as NK/NKT cells is very important for proper DC activation and priming of adaptive anti-tumor immune responses.

Task 5b. DC vaccination optimization by restoration of DC clustering and maturation (months 24-48).

Dendritic cells are the primary antigen presenting cells *in vivo* and their proper activation and maturation are vital for priming potent and effective anti-tumor adaptive immune responses. Novel strategies for inducing immunogenic tumor cells death *in vivo* include the use of thermal ablation or oncolytic viruses armed with GM-CSF, for optimized local recruitment and expansion of the dendritic cells. Cell-associated material released from the dying tumor cells is acquired by dendritic cells migrating to the draining lymph node, where, following proper processing and cross-presentation, tumor antigens are presented for activation of T cell responses. Dysfunctional dendritic cell responses can thus significantly limit the vaccination effect and promote tolerance rather than protective anti-tumor immune responses. Previous studies have identified the MFG-E8-avb3/5 axis in dendritic cells as key for promoting tolerance to apoptotic tumor cell-associated antigens, resulting in the preferential expansion of Tregs over T cell effectors.

In order to antagonize this tolerance promoting mechanism and improve DC functions we decided to investigate whether the circular iRGD peptide (*29, 30*) can be used to enhance DC activation and effector T cell responses in preliminary studies *in vitro*. The iRGD peptide contains the same RGD tri-peptide motif used by MFG-E8 as well as molecules like HMGB1 and osteopontin for binding to avb3/5 integrins, which allows them to modulate the functional status of dendritic cells (*31*) . Integrins are also directly involved in the contacts between cells or between cells and the extra-cellular matrix, thus raising the possibility that the MFG-E8-avb3/5 axis might also be involved in the previously observed tumor-associated defect in normal DC clustering.

Our initial hypothesis that iRGD can be used to block MFG-E8-avb3/5 integrin-mediated tolerance (**Figure 20**), was tested by comparing the effect of MFG-E8 and iRGD on signaling pathways previously reported to be downstream of avb3/5 integrins, including induction of p-Akt, p-Src, p-p65 (NFkB), and p-Stat3. Our preliminary results based on Phosflow analysis of stimulated B16 cells, PBMCs, and DCs indicated that MFG-E8 and iRGD acted as agonists, suggesting that binding of both molecules induced partially overlapping signaling cascades. This finding also raised the question of whether, analogously to MFG-E8, iRGD will be able to promote tolerance rather than protective anti-tumor responses. MFG-E8 and iRGD alone induced very week signaling responses. Previous work, however, has demonstrated that MFG-E8-avb3/5 integrins actually

synergized with other RTK and signaling pathways such as VEGF and IL-6 . Consistent with that, we were able to demonstrate that MFG-E8 and iRGD acted synergistically in augmenting IL-6-p-Akt and LPS-p-p65(NFkB) signaling in dendritic cells (**Figure 21**). Surprisingly, synergistic augmentation of the tolerance-promoting IL-6-Stat-3 signaling pathway was not observed. This effect might be attributed to excessive concentrations of MFG-E8 becoming activating rather than immunosuppressive, a phenomenon also previously reported in the literature (*32*). Importantly, the ability of iRGD to augment p-p65(NFkB) signaling, which is essential for TLR-mediated activation of dendritic cells, strongly argued in favor of the potential use of iRGD as adjuvant and activator of dendritic cell functions, and possibly may restore maturation and clustering which helps illicit proper immune responses. Further work will be necessary to investigate the effects of iRGD on other aspects of dendritic cell activation and function such as up-regulation of MHC II, CD80/CD83/CD86 co-stimulatory molecules, and the balance of pro-inflammatory (IL-1,IL-6,IL-12, and IL-23) versus immunosuppressive cytokines (IL-10 and TGFb).

Task 5c. Optimization of the amplification and effector phases by correcting chronic IL6-mediated defective T cell responses . (month 24-48).

The potential ability of iRGD to partially synergize with IL-6 signaling in DCs led us to further explore of the possibility that iRGD may or may not be able to reverse the dysfunctional responses of T cells from breast cancer patients to IL-6 stimulation. IL-6 signaling is important in driving Th17 and suppressing Treg differentiation as well as in rendering effector T cells resistant to Treg-mediated suppression.

We conducted experiments evaluating the direct effects of iRGD on T cell activation and proliferation in DC-free cultures. CD3+ T cells isolated from the PBMCs of healthy donors were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies in the presence/absence of exogenous IL-2. T cell activation and proliferation after 4 or 7 day time points was evaluated by flowcytometry analysis of CD25 up-regulation and CFSE-dilution, respectively. Results show that treatment with iRGD may augment T cell proliferation, which was associated with up-regulation of CD25 **(**data not shown) and requires more analysis to determine statistical significance. Of note, the effect of iRGD was seen only in the context of combined anti-CD3 and anti-CD28 stimulation suggesting a requirement for proper T cell stimulation signals, rather than a general non-specific T cell activating effect.

Results also indicate a slight enhancement of iRGD-potentiated T cell activation and proliferative responses observed in DC-T cell co-culture experiments. Dendritic cells were generated after 5 day stimulation of purified CD14+ PBMCs in the presence of GM-CSF and IL-4. In such a complex system, however, it is difficult to distinguish whether the observed enhanced responses were mediated through effects of iRGD on DCs or T cells directly. Stimulation of T cells with immature or LPS-matured autologous DCs and with/without soluble anti-CD3 appear to stimulate potent activation of T cells in antigen non-specific way. Although these conditions were appropriate for evaluating the effect of iRGD on the ratio of Teff/Tregs, significant effects on proliferation and T cell activation markers were difficult to see. These conditions were not particularly informative about the potential effects of iRGD on anti-tumor antigen-specific responses *in vivo*.

Task 5d. Testing other strategies and combinations. (month 48-60).

Given that the Teff/Treg ratio has been shown to be the most predictive indicator for the outcome of cancer immunotherapy in patient as well as in animal models, we wanted to establish an informative *in vitro* system to evaluate the effect of different drugs and combination of drugs on DC-driven T cell responses. Dendritic cells and T cells can be co-cultured in autologous or allogeneic settings, the latter stimulating much lower Teff/Treg ratios. Proper activation of T cells required MHC II expression (T2 cells were used as negative control) and additional stimulation with soluble aCD3 antibodies or maturation of the DCs with LPS (**Figure 22**). The potentiating effect of iRGD was observed in both the CD4+ and the CD8+ T cell compartments (**Figure 23**). The effect involved both an enhanced expansion of CD25+Foxp3- effectors as well as a reduction in the frequency of CD25+Foxp3+ Tregs, iRGD improved the Teff/Treg ratio to levels similar to LPS-matured

DCs, underscoring the adjuvant-mimicking effects of iRGD and its potential use to augment anti-tumor adaptive T cell responses *in vivo*.

Further improvements to our system will be made by looking at antigen cross-presentation and the Teff/Treg ratios in murine DC-T cell co-cultures after pulsing the DCs with Ova-expressing tumor cells, and using naïve OT-I/OT-II T cells as responders. This will allow us to establish direct correlations between cross-presentation of tumor-associated antigen, the Teff/Treg ratio and the expansion of antigen-specific T cells. Such a system would be an invaluable tool to identify optimal drug combinations and doses for further studies *in vivo*.

Plans for the next 12 months:

- Methods of enhancing integrated cancer immunotherapy will be investigated further to identify potential tumor eradication techniques coupled with immune-modulatory agents or chemotherapeutics with synergistic effects will be explored.

- Engineered OVA-expressing cells onto B16 melanoma or E0771 GFP/luciferase transfected breast cancer lines to investigate tumor specific T cell responses with OT-I/OT-II transgenic T cells. This will aid in future *in vivo* models to help evaluate tumor specific responses.

- DC vaccination potential will be investigated by combining therapeutic options identified by combined results of all tasks will be moved to *in vivo* analysis once rational approaches are determined *in vitro*.

Supporting Data/Figures:

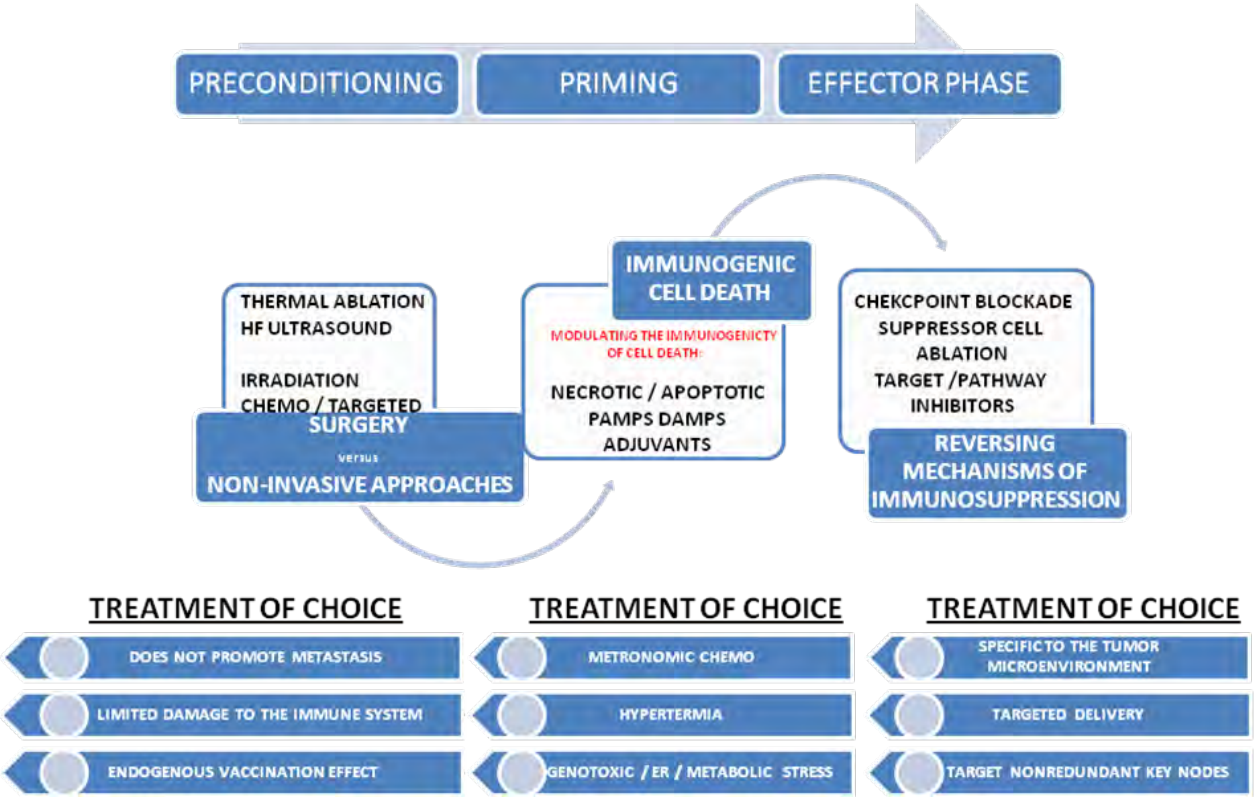


Figure 14: Platform for integrated cancer immunotherapy. Flow chart details the three main prerequisites for proper immune response and the potential approaches to investigate as we move forward with our *in vitro* and *in vivo* assays to develop integrated and combinatorial immunotherapy for cancer.

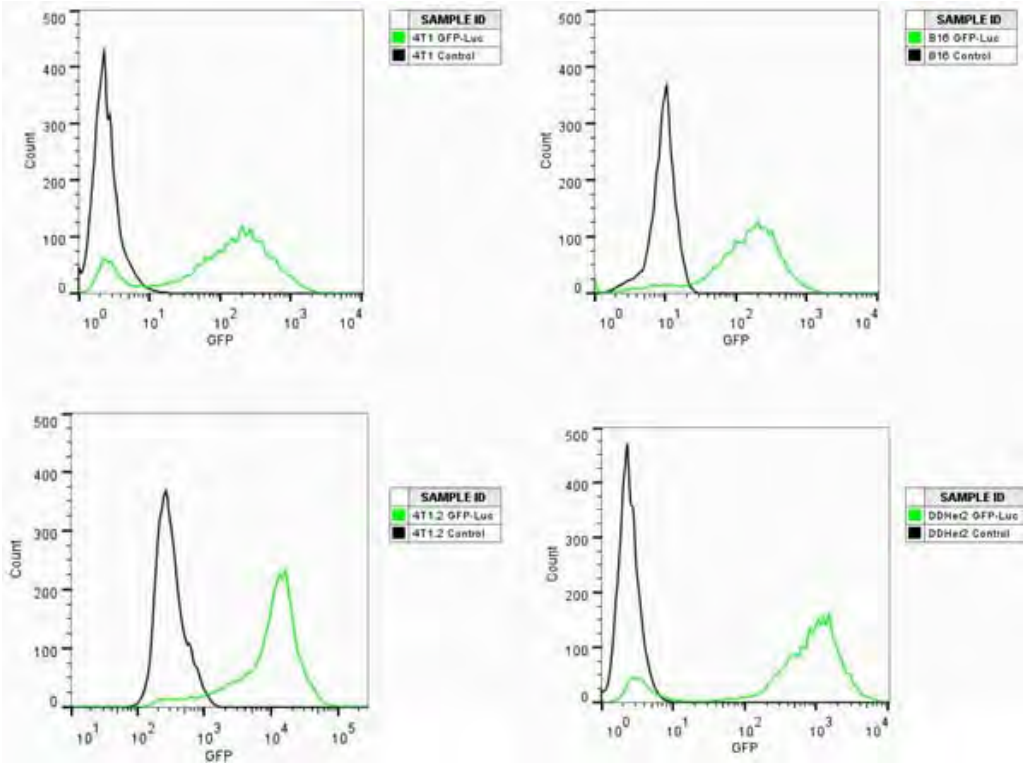


Figure 15: FACS analysis of 4T1, 4T1.2, B16, and DDHer2 murine breast cancer cell lines which were stably transfected with GFP and luciferase via a retroviral vector system.

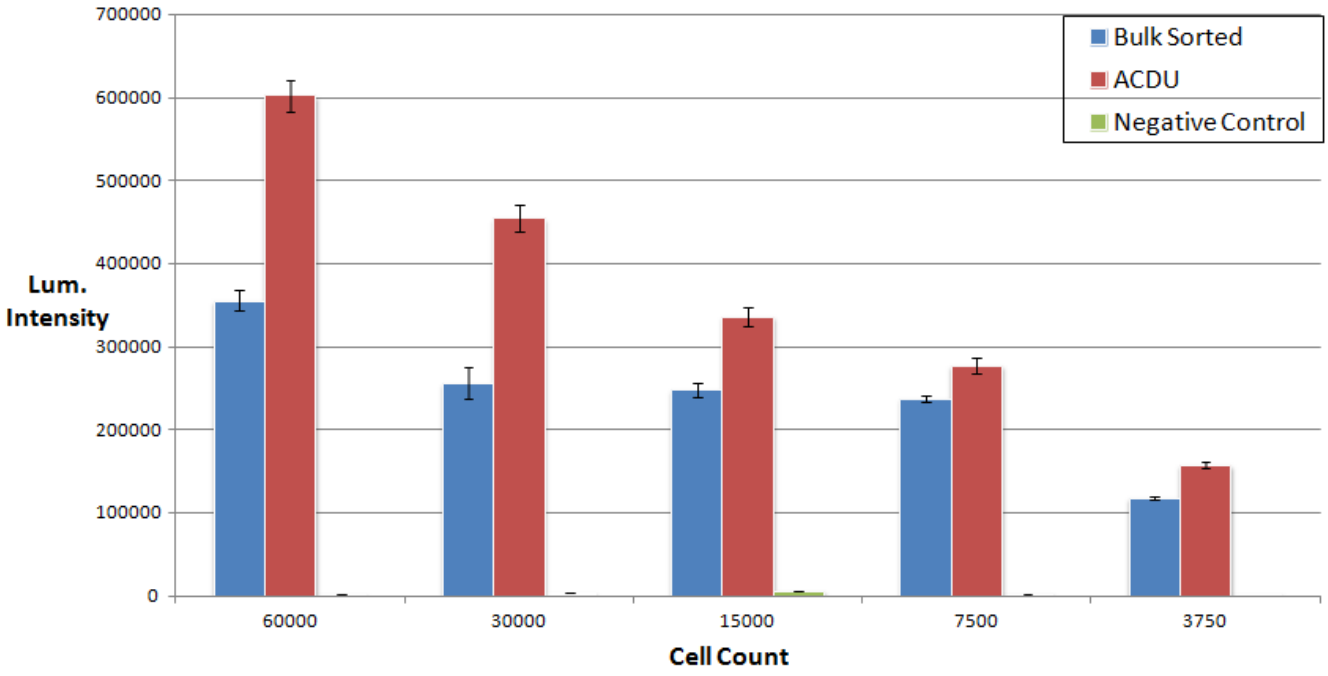


Figure 16: Luciferase expression of stably transfected E0771 breast cancer line. Cell counts for each group are presented on X-axis and Luminescent intensity units on the Y-Axis. Green bar represents negative control non-transfected cells, blue represents bulk population of transfected cells, and red represents cells expanded post FACS cell sort ACDU (Automated Cell Deposition Unit) for highest GFP expression. Cells sorted and then expanded for highest GFP expression (red bar ACDU) correspond with highest luciferase activity and will be used *in vivo.*

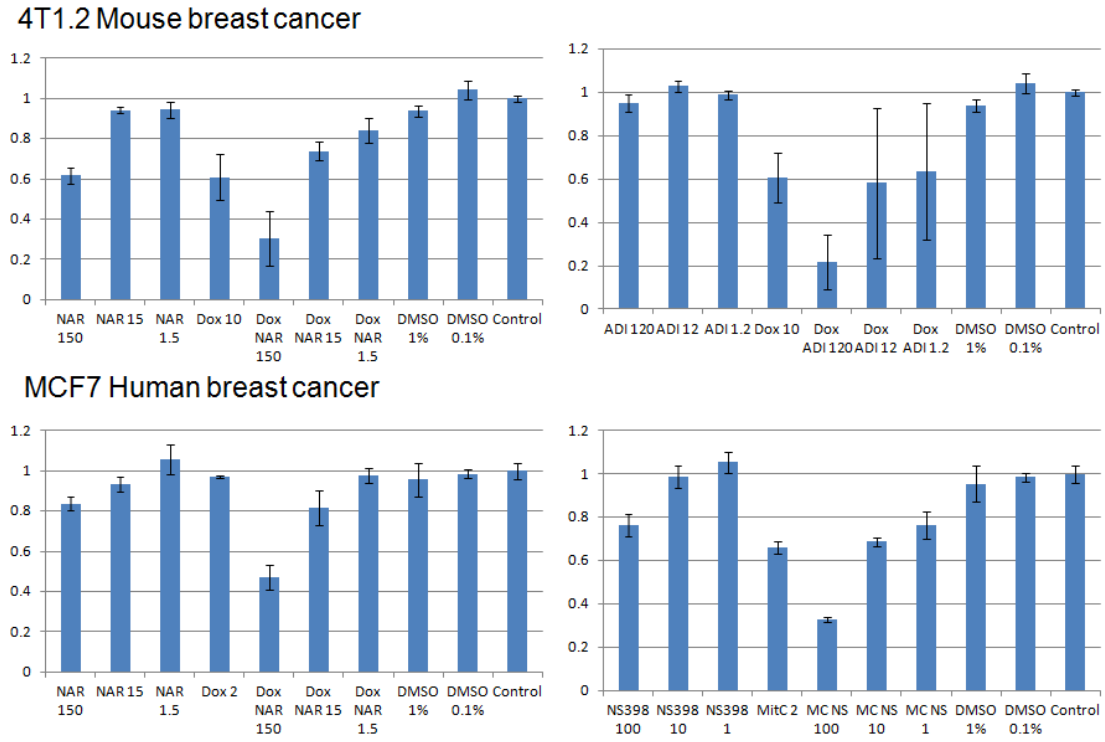


Figure 17: Secondary drugs synergize with Doxorubicin and Mitomycin C chemotherapy in human and mouse breast cancer cell lines. Mouse 4T1.2 (top) and human MCF7 (bottom) breast cancer cells were treated with an IC50 dose of Doxorubicin/Mitomycin C in combination with escalating doses of the secondary drugs naringenin, adiphenine, ivermectin, and ascorbic acid, as indicated. LDH released from 1% Triton X100-lyzed cells was measured with the Promega Cytotox 96 assay and was proportional to the number of live cells. Cell survival is represented normalized to untreated control cells (1), error bars represent standard deviation of triplicate wells.

Synergy Quantification:

Combination Index values were calculated using the Chou-Talalay method: $CI_{A+B} = \frac{D_{A(A+B)}}{D_A} + \frac{D_{B(A+B)}}{D_B}$

- The combination index is used to depict synergism (CI<1), additive effect (CI=1), and antagonism (CI>1). Although other synergy determining systems exist, such as the Bliss model and Loewe additivity, the Chou-Talalay method was used to identify synergy because the previous models do not account for the common sigmoidal shape of dose response curves.

Combination	Cell Line and concentrations in uM	Drug1 Alone (% Live Cells)	Drug2 Alone (% Live Cells)	Combo (% Live Cells)	CI
Doxo+Nar	4T1.2(10,150)	60%	60%	30%	**0.55**
Doxo+Adi	4T1.2(10,120)	54%	95%	21%	**0.12**
Doxo+Nar	MCF7(2,150)	97%	84%	47%	**0.70**
Doxo+Adi	MCF7(2,120)	97%	114% (or near 100%)	94%	1
Mit + NS	4T1.2 (2,100)	54%	76%	58%	**1.75**
Mit + Iver	4T1.2 (2,120)	54%	1%	1%	.95
Mit + NS	MCF7	66%	76%	33%	**0.63**
Mit + Iver	MCF7	66%	7%	8%	1.2

Figure 18: Chemotherapy and Secondary Drug Synergies. The table shows summary of the observed positive/negative synergies calculated using the chemo/secondary drug alone treatments vs. combo treatment.

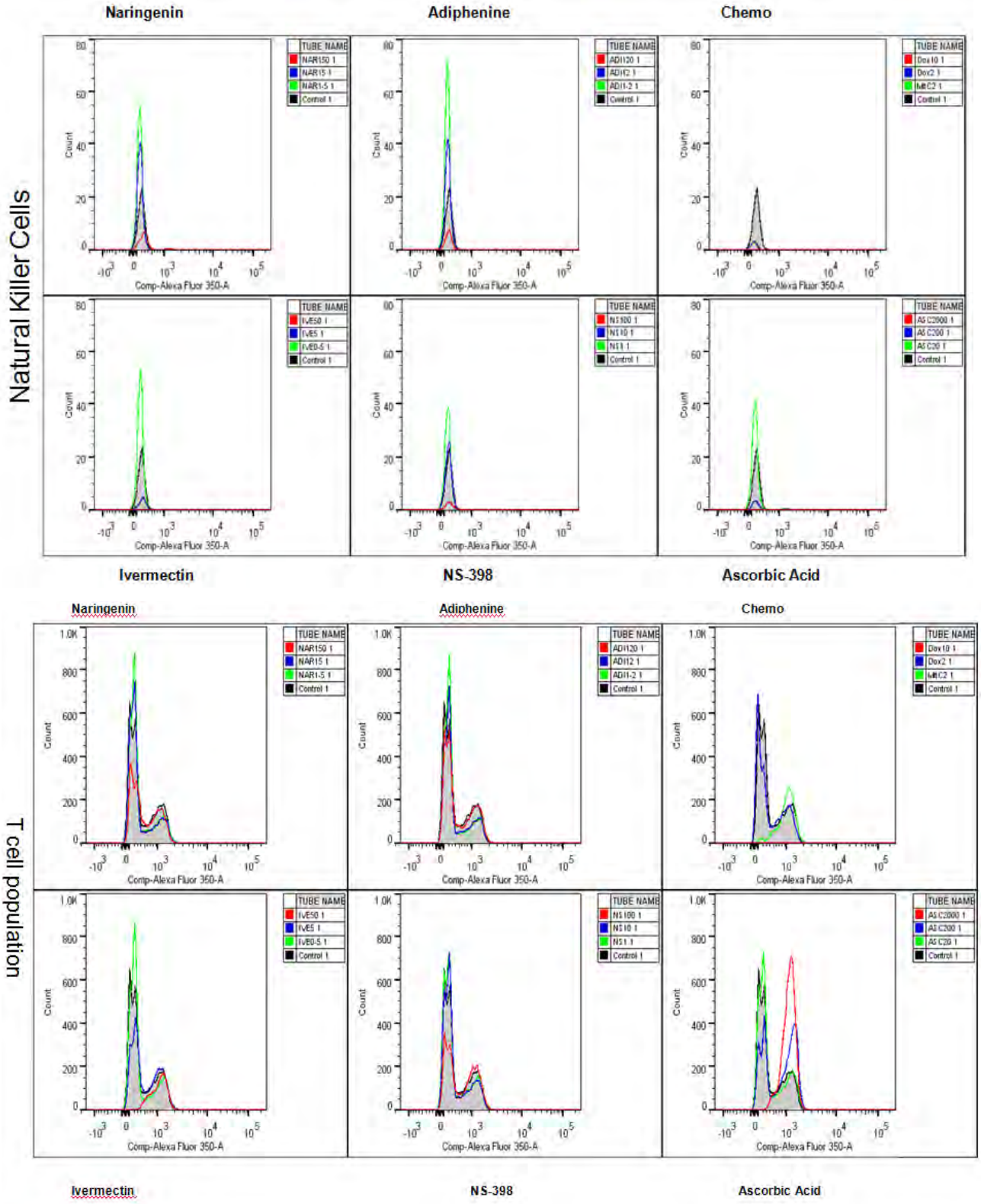


Figure 19: Toxicity of chemo or secondary drugs to human PBMC cells. Natural Killer and T cell subsets from healthy donor PBMCs were incubated for 3 days with Doxorubicin, Mitomycin C, or the secondary drugs Naringenin, Adiphenine, NS-398, Ivermectin, Ascorbic acid, as indicated. PBMCs were stained with fluorescently labeled antibodies to CD19, CD3, CD14, CD16/CD56 and survival of the different cell subsets was evaluated by exclusion/incorporation of the Invitrogen viability probe L23105 blue, 1:1000, with dead cells identified as the AF350-positive population. Flow cytometry overlay histograms show PBMCs treated with Doxorubicin (2 vs. 10 uM), Mitomycin C (2 ug/ml) or escalating (10-fold) doses of secondary drugs (uM as indicated), low (green), medium (blue) and high (red), against untreated controls (black tinted).

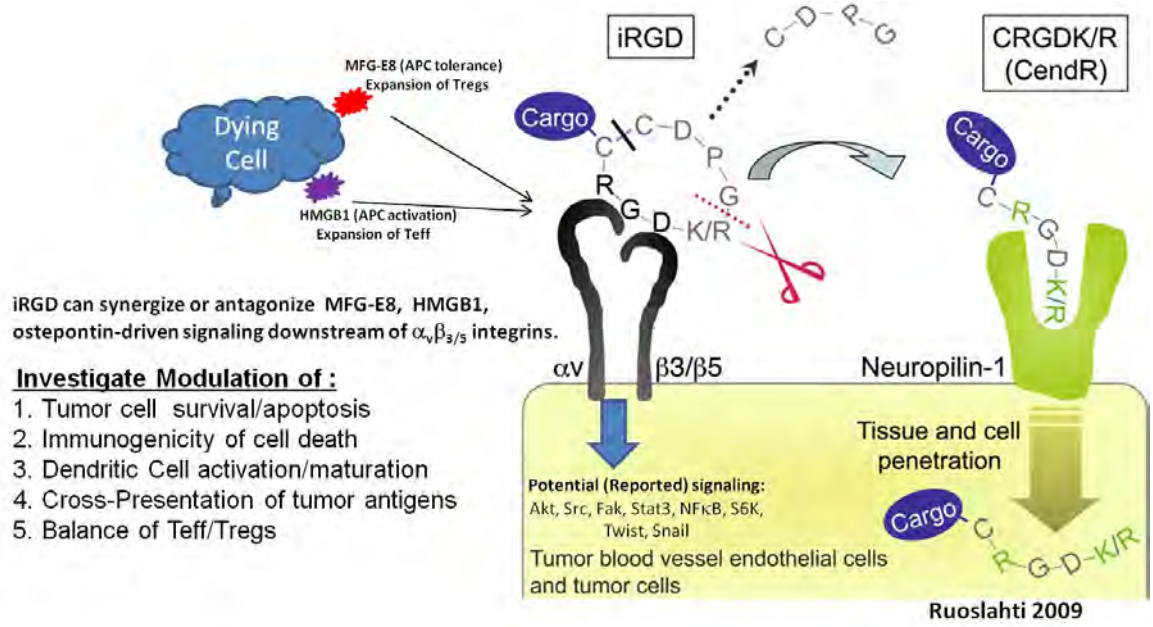


Figure 20: Schematic for iRGD and MFG-E8 Target Cell / Immune Modulating Pathways. Pathways detailed here for iRGD impact tumor cells or associated blood vessel endothelial as well as the impact that MFG-E8 has on these signaling events. Questions regarding these agents impact on immunogenicity, dendritic cell activation/maturation/and potential cross presentation, and Teff/Treg ratios are raised.

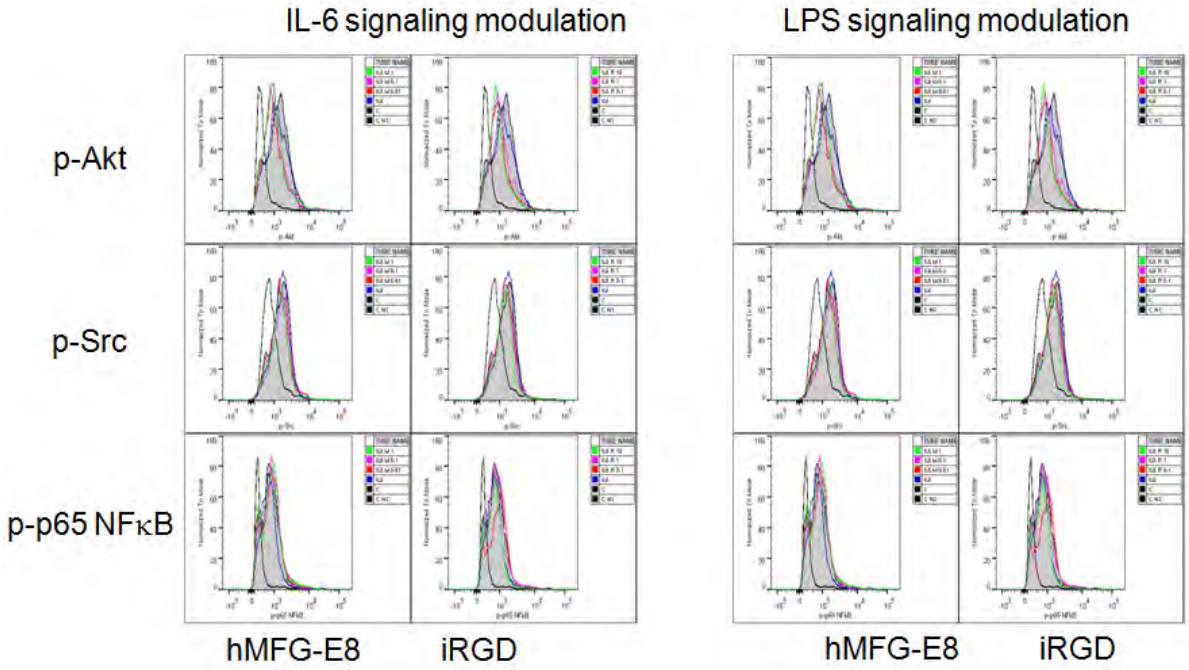


Figure 21: iRGD synergizes with IL-6 and LPS signaling in human PBMC-derived dendritic cells. Human DCs were generated by culturing of CD14+ PBMCs in 50 ng//ml hGM-CSF and 100 ng/ml hIL-4 for 7 days. DCs were plated on 96-well U bottom wells and incubated for 24h in R10 medium supplemented with 100 ng/ml LPS, 100 ng/ml hIL-6, 0.1/1/10 ug/ml iRGD, or 0.01/0.1/1 ug/ml hMFGE8. Cells were fixed in 1.6% PFA for 10 minutes and permeabilized in methanol. Akt, Src, and NFkB signaling was evaluated by intracellular staining with fluorescently labeled antibodies to p-Akt, p-Src, and p-p65, respectively. The overlay histogram plots show signaling in DCs treated with IL-6/LPS alone (blue) or in combination with escalating (10-fold) doses of MFGE8/iRGD - red (low) purple (medium) green (high) vs. untreated control DCs (black tinted) or unstained control (black empty).

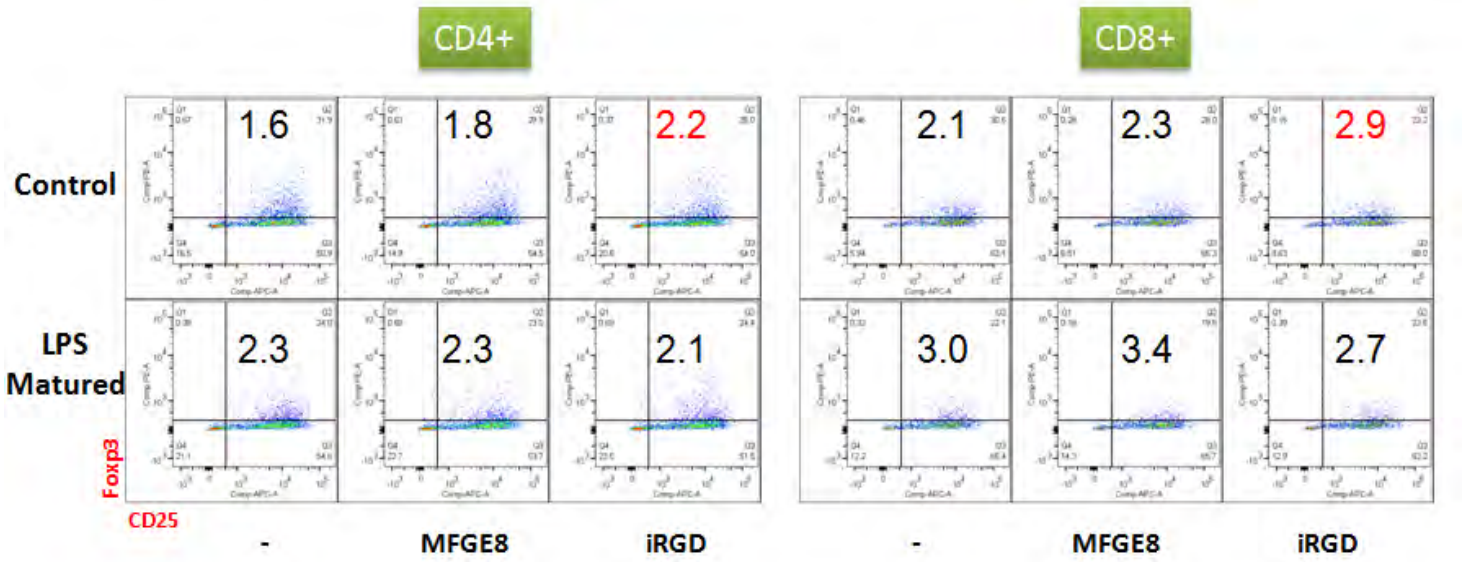
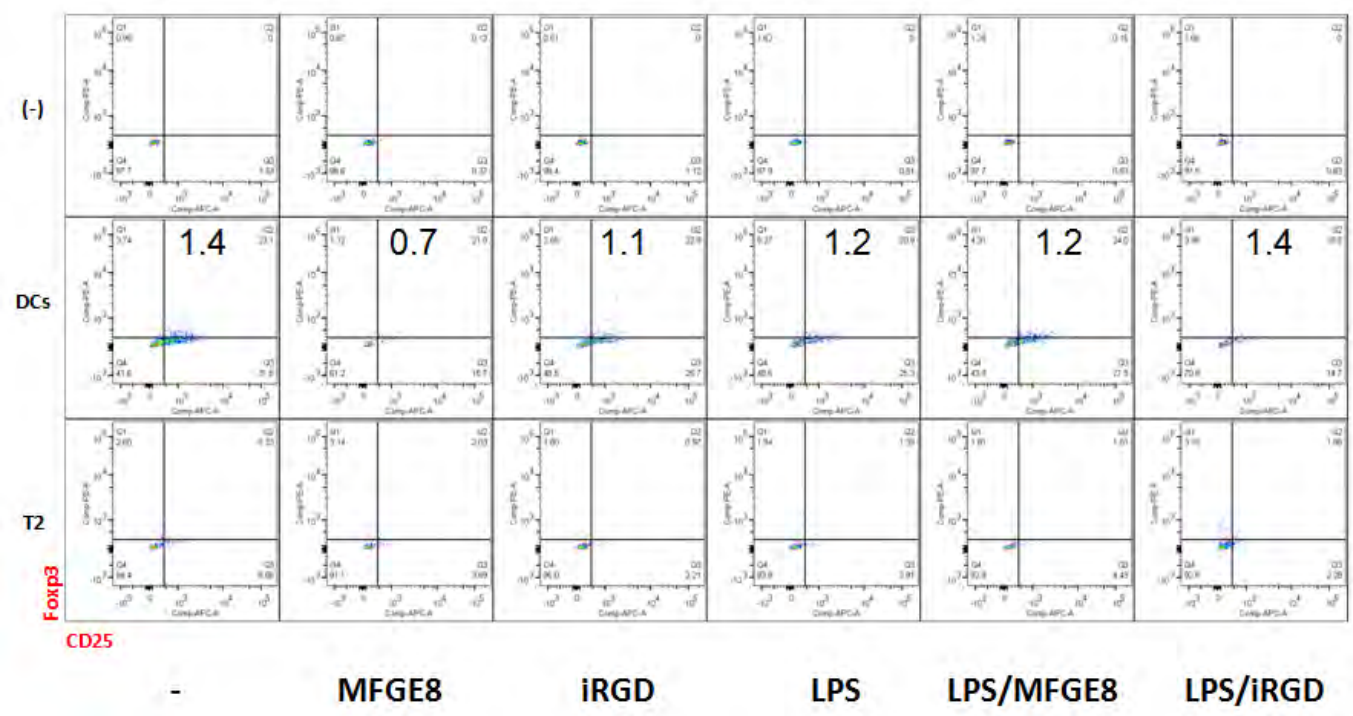


Figure 22: iRGD but not MFGE8 augments the Teff/Treg ratio in the CD4+ and CD8+ T cell compartment. Human DCs were generated by culturing CD14+ PBMCs in 50 ng//ml hGM-CSF and 100 ng/ml hIL-4 for 5 days. Autologous CD3 T cells were stimulated for 4 days with untreated (immature) or LPS – matured DCs (pre-treated with 10 ng/ml LPS for 2 days), and in the presence of 60 ng/ml soluble aCD3 mAbs. Cells were stained with fluorescently labeled antibodies to CD4, CD8, CD25, and Invitrogen fixable viability probe L23105 blue. Staining for Foxp3 was done following fixation and permeabilization using the BD 554655/554723 kits and manufacturer's protocol. The flow cytometry plots indicate the fractions of CD25+Foxp3- Effectors vs. CD25+Foxp3+ Tregs in gated live CD4+ or CD8+ T cells. iRGD enhances the Teff/Treg ratio to levels achieved by LPS-matured DCs.

A)



B)

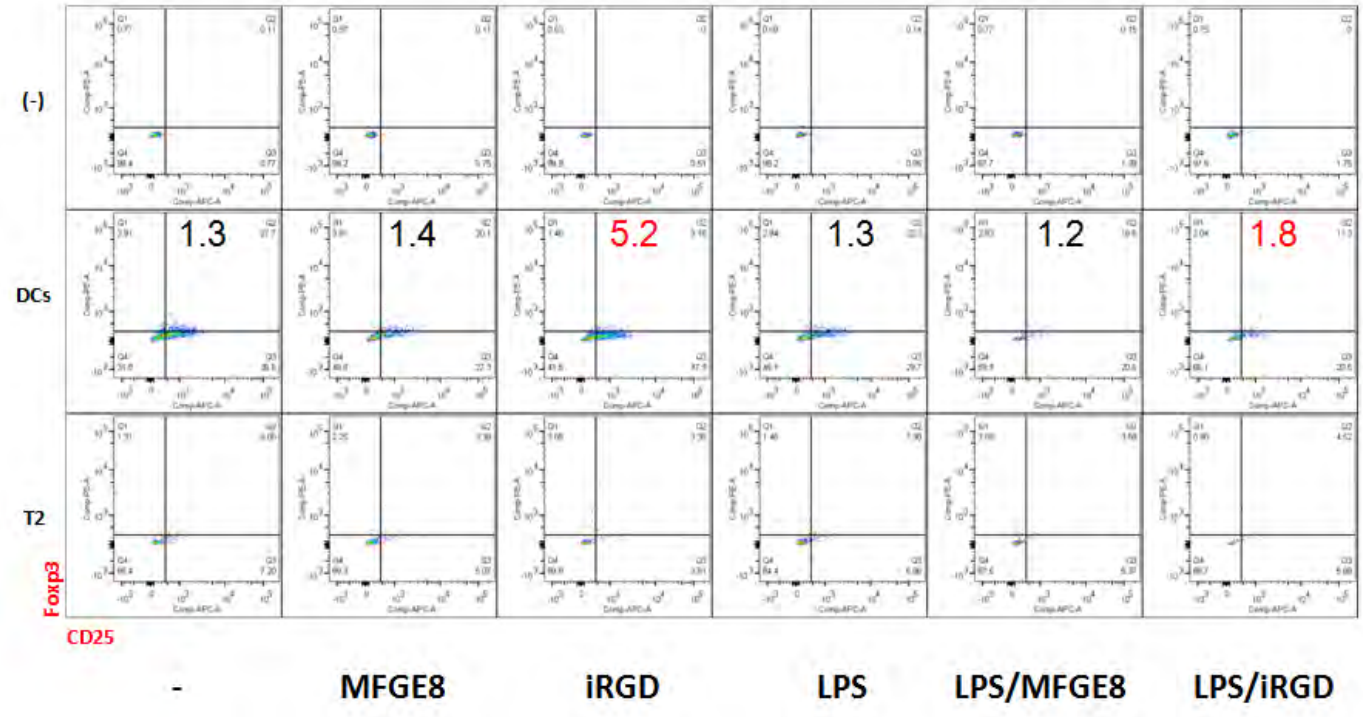


Figure 23: The enhancement in the Teff/Treg ratio is APC-mediated and requires proper T cell stimulation through the TCR (aCD3 plus MHC expression). CD3+ T cells were stimulated with autologous DCs or T2 cells (TAP deficient, MHCII-negative artificial APCs) in (**A**) the absence or (**B**) presence of soluble aCD3 mAbs.

KEY RESEARCH ACCOMPLISHMENTS:

- Optimization of a novel 3D cell culture approach to expand and re-aggregate subpopulations of primary human breast cancer stromal and immune cells, which are critical for supporting or inhibiting DC clustering and maturation as well as cancer growth.

- Histological and FACS based analysis of stromal cells confirms their heavy involvement and frequency within primary and metastatic tumors. Re-creation of these environments has been established and confirm our 3D aggregate method when compared to primary histological samples.

- Separation and isolation techniques of crucial and viable cancer patient derived tumor infiltrating lymphocytes (TILs) has been explored and validated via FACS analysis. These cells will be essential when incorporated into our *in vitro* assays for determining role in DC clustering/maturation or interactions with tumor associated stroma.

- Banked RNA of various stromal cell types from both healthy and tumor associated tissues to determine molecules or genetic expression profiles for future analysis.

- Isolated and expanded PBMC derived DCs for future use in DC related assays.

- Validated initial stromal and DC-3D aggregate cultures to explore clustering in a synthetic system and observed similar clustering to what was previously established.

- Identified regulation effect of chronic IL-6 stimulation on IL-6 & IL-27 signaling, GP130 and IL-6Rα expression, and STAT1 and STAT3 expression levels by chronic IL-6 stimulation.

- Provided results on enhancement of PBMC and T cell survival and proliferation when treated with IL-6.

- Effectiveness of anti-IL-6 treatment on the regulation of GP130 and IL-6Rα expression, STAT1 and STAT3 expression, and IL-6 & IL-27 signaling was assessed and point towards potential use for anti-IL-6 in modulating tumor microenvironment.

- 4T1, 4T1.2, E0771 and B16 cells were stably transfected with retroviral vectors to express GFP and luciferase, which was documented by flow cytometry analysis and luminescent analysis.

- Using gene expression data from the MCF7 human breast cancer line we have identified several combinations of chemotherapeutic (doxorubicin, mitomycin C, paclitaxel) and secondary drugs (Naringenin, Adiphenine, NS-398, Ivermectin, Ascorbic Acid) predicted to have synergistic anti-tumor effects.

- Anti-tumor drug combination synergistic effects were observed including some which indicate minimal toxicity to normal human PBMCs.

- Demonstrated that MFG-E8 and iRGD act synergistically in augmenting IL-6-p-Akt and LPS-p-p65(NFkB) signaling in dendritic cells and may influence functionality.

- The potentiating effect of iRGD was observed in both the CD4+ and the CD8+ T cell compartments and the effect involved both an enhanced expansion of CD25+Foxp3- effectors as well as a reduction in the frequency of CD25+Foxp3+ Tregs, iRGD slightly improved the Teff/Treg ratio to levels similar to LPS-matured DCs.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

None at this time – studies ongoing.

CONCLUSION:

In Year One, I have built a strong research team for this project. This includes staff scientist Dr. Brile Chung, PhD postdoctoral fellows Dr. Dobrin Draganov and Dr. Neta Zuckerman, research associates John Murad and Grace Jimenez. We worked closely with the CoH IRB office on a human subject's protocol which has been approved. We have begun to develop an in-depth understanding of the immune system within the setting of the tumor microenvironment. We have made progress in developing methods to better analyze the relationships between primary tumor and metastatic growths with their surrounding microenvironment and implications for immune response and dendritic cell function *in vitro* using 3D microculture techniques. We have further investigated the roles of specific cytokines and potential blockade of cytokines on restoring immune signaling and tested alternative methods of combinatorial drugs in attempts to eradicate cancer cells while preserving the immune system. We are well positioned to gain further insights in the next 12 months that will aid in our goal of restoring long term immune function in breast cancer patients to optimal levels, and subsequently eradicate metastases to prevent relapse in breast cancer patients.

APPENDICES:

None at this time.

PERSONNEL:

Peter P. Lee, MD – PI	35% Effort
Brile Chung, PhD – Staff Scientist	100% Effort
Dobrin Draganov, PhD – Post Doctoral Fellow	100% Effort
Neta Zuckerman, PhD – Post Doctoral Fellow	50% Effort
John P. Murad, M.S. – Research Associate I	100% Effort
Grace Jimenez – Research Technician	100% Effort

REFERENCES:

1. C. N. Baxevanis *et al.*, Tumor specific cytolysis by tumor infiltrating lymphocytes in breast cancer. *Cancer* **74**, 1275 (Aug 15, 1994).
2. G. B. Cannon, R. Pomerantz, Cell-mediated immune responses--prognostic indicators of survival from breast cancer. *Int J Cancer* **44**, 995 (Dec 15, 1989).
3. J. L. McCoy, R. Rucker, J. A. Petros, Cell-mediated immunity to tumor-associated antigens is a better predictor of survival in early stage breast cancer than stage, grade or lymph node status. *Breast Cancer Res Treat* **60**, 227 (Apr, 2000).
4. H. E. Kohrt *et al.*, Profile of immune cells in axillary lymph nodes predicts disease-free survival in breast cancer. *PLoS Med* **2**, e284 (Sep, 2005).
5. B. Weigelt, A. T. Lo, C. C. Park, J. W. Gray, M. J. Bissell, HER2 signaling pathway activation and response of breast cancer cells to HER2-targeting agents is dependent strongly on the 3D microenvironment. *Breast Cancer Res Treat* **122**, 35 (Jul, 2010).
6. M. Vanneman, G. Dranoff, Combining immunotherapy and targeted therapies in cancer treatment. *Nature reviews. Cancer* **12**, 237 (Apr, 2012).
7. B. Ljujic *et al.*, Human mesenchymal stem cells creating an immunosuppressive environment and promote breast cancer in mice. *Scientific reports* **3**, 2298 (Jul 29, 2013).
8. T. D. Tlsty, L. M. Coussens, Tumor stroma and regulation of cancer development. *Annual review of pathology* **1**, 119 (2006).
9. M. Mohtashami, D. K. Shah, K. Kianizad, G. Awong, J. C. Zuniga-Pflucker, Induction of T-cell development by Delta-like 4-expressing fibroblasts. *International immunology*, (Aug 29, 2013).
10. N. S. Zuckerman, Y. Noam, A. J. Goldsmith, P. P. Lee, A self-directed method for cell-type identification and separation of gene expression microarrays. *PLoS computational biology* **9**, e1003189 (Aug, 2013).
11. P. C. Heinrich, I. Behrmann, G. Muller-Newen, F. Schaper, L. Graeve, Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *Biochem J* **334 (Pt 2)**, 297 (Sep 1, 1998).
12. H. Korkaya, S. Liu, M. S. Wicha, Regulation of Cancer Stem Cells by Cytokine Networks: Attacking Cancers Inflammatory Roots. *Clin Cancer Res*, (Jun 17, 2011).
13. H. Knupfer, R. Preiss, Significance of interleukin-6 (IL-6) in breast cancer (review). *Breast Cancer Res Treat* **102**, 129 (Apr, 2007).
14. R. Salgado *et al.*, Circulating interleukin-6 predicts survival in patients with metastatic breast cancer. *Int J Cancer* **103**, 642 (Feb 20, 2003).
15. A. Kimura, T. Kishimoto, IL-6: regulator of Treg/Th17 balance. *Eur J Immunol* **40**, 1830 (Jul, 2010).
16. X. Zhang, T. Goel, L. L. Goodfield, S. J. Muse, E. T. Harvill, Decreased leukocyte accumulation and delayed Bordetella pertussis clearance in IL-6-/- mice. *J Immunol* **186**, 4895 (Apr 15, 2011).
17. M. Kopf *et al.*, Impaired immune and acute-phase responses in interleukin-6-deficient mice. *Nature* **368**, 339 (Mar 24, 1994).
18. S. Pflanz *et al.*, WSX-1 and glycoprotein 130 constitute a signal-transducing receptor for IL-27. *J Immunol* **172**, 2225 (Feb 15, 2004).
19. E. Tassi *et al.*, Non-redundant role for IL-12 and IL-27 in modulating Th2 polarization of carcinoembryonic antigen specific CD4 T cells from pancreatic cancer patients. *PLoS One* **4**, e7234 (2009).
20. T. Yoshimoto, K. Yasuda, J. Mizuguchi, K. Nakanishi, IL-27 suppresses Th2 cell development and Th2 cytokines production from polarized Th2 cells: a novel therapeutic way for Th2-mediated allergic inflammation. *J Immunol* **179**, 4415 (Oct 1, 2007).
21. S. Pflanz *et al.*, IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4(+) T cells. *Immunity* **16**, 779 (Jun, 2002).
22. N. Morishima *et al.*, A pivotal role for interleukin-27 in CD8+ T cell functions and generation of cytotoxic T lymphocytes. *J Biomed Biotechnol* **2010**, 605483 (2010).
23. N. Morishima *et al.*, Augmentation of effector CD8+ T cell generation with enhanced granzyme B expression by IL-27. *J Immunol* **175**, 1686 (Aug 1, 2005).

24. K. P. Strauss, Implementing the telecommunications provisions. *Milbank Q* **69 Suppl 1-2**, 238 (1991).
25. G. Regis, S. Pensa, D. Boselli, F. Novelli, V. Poli, Ups and downs: the STAT1:STAT3 seesaw of Interferon and gp130 receptor signalling. *Semin Cell Dev Biol* **19**, 351 (Aug, 2008).
26. L. Y. Kong *et al.*, A novel phosphorylated STAT3 inhibitor enhances T cell cytotoxicity against melanoma through inhibition of regulatory T cells. *Cancer Immunol Immunother* **58**, 1023 (Jul, 2009).
27. M. Kortylewski *et al.*, Inhibiting Stat3 signaling in the hematopoietic system elicits multicomponent antitumor immunity. *Nat Med* **11**, 1314 (Dec, 2005).
28. L. Anasagasti-Angulo, Y. Garcia-Vega, S. Barcelona-Perez, P. Lopez-Saura, I. Bello-Rivero, Treatment of advanced, recurrent, resistant to previous treatments basal and squamous cell skin carcinomas with a synergistic formulation of interferons. Open, prospective study. *BMC Cancer* **9**, 262 (2009).
29. E. Ruoslahti, The RGD story: a personal account. *Matrix biology : journal of the International Society for Matrix Biology* **22**, 459 (Nov, 2003).
30. L. Alberici *et al.*, De novo design of a tumor-penetrating peptide. *Cancer research* **73**, 804 (Jan 15, 2013).
31. M. Jinushi *et al.*, MFG-E8-mediated uptake of apoptotic cells by APCs links the pro- and antiinflammatory activities of GM-CSF. *J Clin Invest* **117**, 1902 (Jul, 2007).
32. H. Yamaguchi *et al.*, Milk fat globule EGF factor 8 in the serum of human patients of systemic lupus erythematosus. *J Leukoc Biol* **83**, 1300 (May, 2008).